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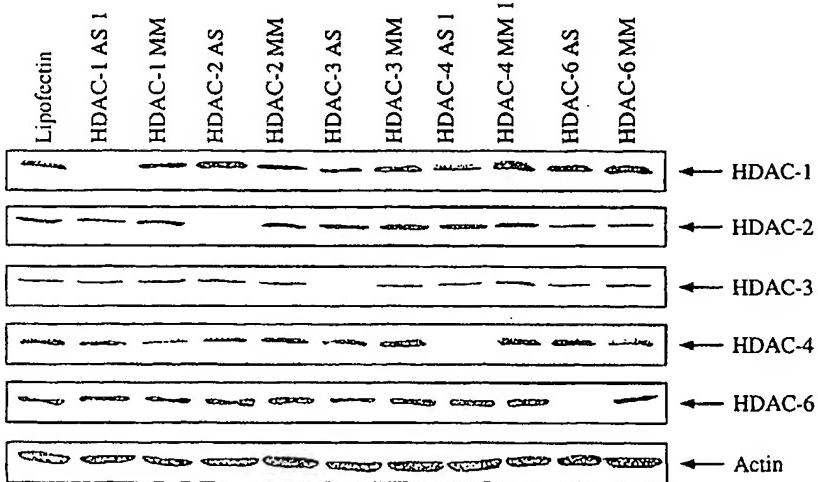
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[Continued on next page]

(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS



AS = Antisense

MM = Mismatch

NS = Non-specific control

3 day treatment

Oligonucleotide cone – 50nM

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(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

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BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the fields of inhibition of histone deacetylase expression and enzymatic activity.

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Summary of the Related Art

In eukaryotic cells, nuclear DNA associates with histones to form a compact complex called chromatin. The histones constitute a family of basic proteins which are generally highly conserved across eukaryotic species. The core histones, termed H2A, H2B, H3, and H4, associate to form a protein core. DNA winds around this protein core, with the basic amino acids of the histones interacting with the negatively charged phosphate groups of the DNA. Approximately 146 base pairs of DNA wrap around a histone core to make up a nucleosome particle, the repeating structural motif of chromatin.

Csordas, *Biochem. J.*, 286: 23-38 (1990) teaches that histones are subject to posttranslational acetylation of the epsilon-amino groups of N-terminal lysine residues, a reaction that is catalyzed by histone acetyl transferase (HAT1). Acetylation neutralizes the positive charge of the lysine side chain, and is thought to impact chromatin structure. Indeed, Taunton *et al.*, *Science*, 272: 408-411 (1996), teaches that access of transcription factors to chromatin templates is enhanced by histone hyperacetylation. Taunton *et al.* further teaches that an enrichment in underacetylated histone H4 has been found in transcriptionally silent regions of the genome.

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Recently, there has been interest in the role of histone deacetylase (HDAC) in gene expression. Sanches Del Pino *et al.*, *Biochem. J.* 303: 723-729 (1994) discloses a partially purified yeast HDAC activity. Taunton *et al.* (*supra*) discloses a human HDAC that is related to a yeast transcriptional regulator and suggests that this protein may be a key regulator of eukaryotic transcription.

Known inhibitors of mammalian HDAC have been used to probe the role of HDAC in gene regulation. Yoshida *et al.*, *J. Biol. Chem.* 265: 17174-17179 (1990) discloses that (R)-Trichostatin A (TSA) is a potent inhibitor of mammalian HDAC. Yoshida *et al.*, *Cancer Research* 47: 3688-3691 (1987) discloses that TSA is a potent inducer of differentiation in murine erythroleukemia cells.

More recently, it has been discovered that the HDAC activity is actually provided by a set of discrete HDAC enzyme isoforms. Grozinger *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 96: 4868-4873 (1999), teaches that HDACs may be divided into two classes, the first represented by yeast Rpd3-like proteins, and the second represented by yeast Hda1-like proteins. Grozinger *et al.* also teaches that the human HDAC1, HDAC2, and HDAC3 proteins are members of the first class of HDACs, and discloses new proteins, named HDAC4, HDAC5, and HDAC6, which are members of the second class of HDACs. Kao *et al.*, *Gene & Development* 14: 55-66 (2000), discloses an additional member of this second class, called HDAC-7. More recently, Hu, E. *et al.* *J. Bio. Chem.* 275:15254-13264 (2000) disclosed the newest member of the first class of histone deacetylases, HDAC-8. It has been unclear what roles these individual HDAC enzymes play.

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The known inhibitors of histone deacetylase are all small molecules that inhibit histone deacetylase activity at the protein level. Moreover, all of the known histone deacetylase inhibitors are non-specific for a particular histone deacetylase isoform, and more or less inhibit all members of both
5 the histone deacetylase families equally.

Therefore, there remains a need to develop reagents for inhibiting specific histone deacetylase isoforms. There is also a need for the development of methods for using these reagents to identify and inhibit specific histone deacetylase isoforms involved in tumorigenesis.

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BRIEF SUMMARY OF THE INVENTION

The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The inventors have discovered new agents that inhibit specific HDAC isoforms. Accordingly, in a first aspect, the invention provides agents that inhibit one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms. Such specific HDAC isoforms
15 include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. Non-limiting examples of the new agents include antisense oligonucleotides (oligos) and small molecule inhibitors specific for one or more HDAC isoforms but less than all HDAC isoforms.

20 The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the
25 invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

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In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding that histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (e.g., a gene), cDNA, or RNA. In some 5 embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC isoform. In other embodiments, the oligonucleotide inhibits translation of the histone deacetylase isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Particularly preferred embodiments include antisense 10 oligonucleotides directed to HDAC-1 and/or HDAC-4.

In yet other embodiments of the first aspect, the agent that inhibits a specific HDAC isoform is a small molecule inhibitor that inhibits the activity of one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms.

15 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell, comprising contacting the cell with an agent of the first aspect of the invention. In other preferred embodiments, the agent is an antisense oligonucleotide. In certain preferred embodiments, the agent is a small 20 molecule inhibitor. In other certain preferred embodiments of the second aspect of the invention, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method of the second aspect of the 25 invention further comprises contacting the cell with a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of one or more specific histone deacetylase isoforms. In still yet other preferred embodiments of the second aspect of the invention, the method comprises an agent of the first aspect of the invention which is a

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combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In
5 other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4. In some embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide.

In a third aspect, the invention provides a method for inhibiting
10 neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain
15 preferred embodiments, the agent is a small molecule inhibitor which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain preferred embodiments of the this aspect of the invention, cell proliferation
is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In other certain embodiments, the agent is a small molecule inhibitor of the first aspect of the invention which is combined with a pharmaceutically acceptable carrier and administered for
20 a therapeutically effective period of time. In still yet other preferred embodiments of the third aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred
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embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

- 5 In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone
- 10 deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that
- 15 inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is
- 20 a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In
- 25 certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of

5 differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is an small molecule inhibitor of

10 the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first

15 aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting

20 neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for

25 a therapeutically effective period of time.

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In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, 5 HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide 15 from the first aspect of the invention that inhibits expression of a specific histone deacetylase isoform, a small molecule inhibitor from the first aspect of the invention that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a small molecule that inhibits a DNA methyltransferase. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents 20 selected from the group are operably associated. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 25 and/or HDAC-4.

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- In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation, comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of
- 5 proliferation or differentiation. In certain embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In preferred embodiments, the cell proliferation is neoplasia. In still yet other preferred embodiments of the
- 10 this aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5,
- 15 HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic diagram providing the amino acid sequence of HDAC-1, as provided in GenBank Accession No. AAC50475 (SEQ ID NO:1).

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Figure 1B is a schematic diagram providing the nucleic acid sequence of HDAC-1, as provided in GenBank Accession No. U50079 (SEQ ID NO:2).

10 Figure 2A is a schematic diagram providing the amino acid sequence of HDAC-2, as provided in GenBank Accession No. AAC50814 (SEQ ID NO:3).

15 Figure 2B is a schematic diagram providing the nucleic acid sequence of HDAC-2, as provided in GenBank Accession No. U31814 (SEQ ID NO:4).

20 Figure 3A is a schematic diagram providing the amino acid sequence of HDAC-3, as provided in GenBank Accession No. AAB88241 (SEQ ID NO:5).

Figure 3B is a schematic diagram providing the nucleic acid sequence of HDAC-3, as provided in GenBank Accession No. U75697 (SEQ ID NO:6).

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Figure 4A is a schematic diagram providing the amino acid sequence of HDAC-4, as provided in GenBank Accession No. BAA22957 (SEQ ID NO:7).

5 Figure 4B is a schematic diagram providing the nucleic acid sequence of HDAC-4, as provided in GenBank Accession No. AB006626 (SEQ ID NO:8).

10 Figure 5A is a schematic diagram providing the amino acid sequence of HDAC-5, as provided in GenBank Accession No. BAA25526 (SEQ ID NO:9).

15 Figure 5B is a schematic diagram providing the nucleic acid sequence of HDAC-5 as provided in GenBank Accession No. AB011172 (SEQ ID NO:10).

Figure 6A is a schematic diagram providing the amino acid sequence of human HDAC-6, as provided in GenBank Accession No. AAD29048 (SEQ ID NO:11).

20 Figure 6B is a schematic diagram providing the nucleic acid sequence of human HDAC-6, as provided in GenBank Accession No. AJ011972 (SEQ ID NO:12).

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Figure 7A is a schematic diagram providing the amino acid sequence of human HDAC-7, as provided in GenBank Accession No. AAF63491.1 (SEQ ID NO:13).

5 Figure 7B is a schematic diagram providing the nucleic acid sequence of human HDAC-7, as provided in GenBank Accession No. AF239243 (SEQ ID NO:14).

10 Figure 8A is a schematic diagram providing the amino acid sequence of human HDAC-8, as provided in GenBank Accession No. AAF73076.1 (SEQ ID NO:15).

15 Figure 8B is a schematic diagram providing the nucleic acid sequence of human HDAC-8, as provided in GenBank Accession No. AF230097 (SEQ ID NO:16).

20 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-1 AS1 antisense oligonucleotide on HDAC-1 mRNA expression in human A549 cells.

Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-2 AS antisense oligonucleotide on HDAC-2 mRNA expression in human A549 cells.

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Figure 9C is a representation of a Northern blot demonstrating the effect of HDAC-6 AS antisense oligonucleotide on HDAC-6 mRNA expression in human A549 cells.

5 Figure 9D is a representation of a Northern blot demonstrating the effect of HDAC-3 AS antisense oligonucleotide on HDAC-3 mRNA expression in human A549 cells.

10 Figure 9E is a representation of a Northern blot demonstrating the effect of an HDAC-4 antisense oligonucleotide (AS1) on HDAC-4 mRNA expression in human A549 cells.

15 Figure 9F is a representation of a Northern blot demonstrating the dose-dependent effect of an HDAC-4 antisense oligonucleotide (AS2) on HDAC-4 mRNA expression in human A549 cells.

Figure 9G is a representation of a Northern blot demonstrating the effect of an HDAC-5 antisense oligonucleotide (AS) on HDAC-5 mRNA expression in human A549 cells.

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Figure 9H is a representation of a Northern blot demonstrating the effect of an HDAC-7 antisense oligonucleotide (AS) on HDAC-7 mRNA expression in human A549 cells.

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Figure 9I is a representation of a Northern blot demonstrating the dose-dependent effect of HDAC-8 antisense oligonucleotides (AS1 and AS2) on HDAC-8 mRNA expression in human A549 cells.

5 Figure 10A is a representation of a Western blot demonstrating the effect of HDAC isotype-specific antisense oligos on HDAC isotype protein expression in human A549 cells.

10 Figure 10B is a representation of a Western blot demonstrating the dose-dependent effect of the HDAC-1 isotype-specific antisense oligo (AS1 and AS2) on HDAC isotype protein expression in human A549 cells.

15 Figure 10C is a representation of a Western blot demonstrating the effect of HDAC-4 isotype-specific antisense oligonucleotide (AS2) on HDAC isotype protein expression in human A549 cells.

Figure 11A is a graphic representation demonstrating the apoptotic effect of HDAC isotype-specific antisense oligos on human A549 cancer cells.

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Figure 12A is a graphic representation demonstrating the effect of HDAC-1 AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

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Figure 12B is a graphic representation demonstrating the effect of HDAC-8 specific AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

5 Figure 13 is a graphic representation demonstrating the cell cycle blocking effect of HDAC specific antisense oligonucleotides on human A549 cancer cells.

10 Figure 14 is a representation of an RNase protection assay demonstrating the effect of HDAC isotype-specific antisense oligonucleotides on HDAC isotype mRNA expression in human A549 cells.

15 Figure 15 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-4 AS1 antisense oligonucleotide induces the expression of the p21 protein.

20 Figure 16 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-1 antisense oligonucleotides (AS1 and AS2) represses the expression of the cyclin B1 and cyclin A genes.

25 Figure 17 shows plating data demonstrating the ability of antisense oligonucleotides complementary to HDAC-1 to inhibit growth in soft agar of A549 cells far more than can antisense oligonucleotides complementary to HDAC-2, HDAC-6 or mismatched controls.

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Figure 18 is a representation of a Western blot demonstrating that treatment of human A549 cells with the small molecule inhibitor Compound 3 (Table 2) induces the expression of the p21 protein and represses the expression of the cyclin B1 and cyclin A genes.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides methods and reagents for inhibiting specific histone deacetylase isoforms (HDAC) by inhibiting expression at the nucleic acid level or protein activity at the enzymatic level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the
15 same extent as if each was specifically and individually indicated to be incorporated by reference.

In a first aspect, the invention provides agents that inhibit one or more histone deacetylase isoform, but less than all specific histone deacetylase isoforms. As used herein interchangeably, the terms "histone deacetylase", "HDAC", "histone deacetylase isoform", "HDAC isoform" and similar terms are intended to refer to any one of a family of enzymes that remove acetyl groups from the epsilon-amino groups of lysine residues at the N-terminus of a histone. Unless otherwise indicated by context, the term "histone" is meant to refer to any histone protein, including H1, H2A,
20 H2B, H3, and H4, from any species. Preferred histone deacetylase isoforms include class I and class II enzymes. Specific HDACs include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6,
25 HDAC-7 and HDAC-8. By way of non-limiting example, useful agents that

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inhibit one or more histone deacetylase isoforms, but less than all specific histone deacetylase isoforms, include antisense oligonucleotides and small molecule inhibitors.

The present inventors have surprisingly discovered that specific
5 inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the invention, the histone deacetylase isoform that is inhibited is HDAC-1
10 and/or HDAC-4.

Preferred agents that inhibit HDAC-1 and/or HDAC-4 dramatically inhibit growth of human cancer cells, independent of p53 status. These agents significantly induce apoptosis in the cancer cells and cause dramatic growth arrest. They also can induce transcription of tumor suppressor
15 genes, such as p21^{WAF1}, p57^{KIP2}, GADD153 and GADD45. Finally, they exhibit both *in vitro* and *in vivo* anti-tumor activity. Inhibitory agents that achieve one or more of these results are considered within the scope of this aspect of the invention. By way of non-limiting example, antisense oligonucleotides and/or small molecule inhibitors of HDAC-1 and/or
20 HDAC-4 are useful for the invention.

In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding a specific histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (e.g., a gene), cDNA, or RNA. In
25 other embodiments, the oligonucleotide ultimately inhibits translation of the histone deacetylase. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Preferred antisense

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oligonucleotides have potent and specific antisense activity at nanomolar concentrations.

- The antisense oligonucleotides according to the invention are complementary to a region of RNA or double-stranded DNA that encodes
- 5 a portion of one or more histone deacetylase isoform (taking into account that homology between different isoforms may allow a single antisense oligonucleotide to be complementary to a portion of more than one isoform).

- For purposes of the invention, the term "complementary" means
- 10 having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base
- 15 stacking can lead to hybridization. As a practical matter, such hybridization can be inferred from the observation of specific gene expression inhibition, which may be at the level of transcription or translation (or both).

- For purposes of the invention, the term "oligonucleotide" includes
- 20 polymers of two or more deoxyribonucleosides, ribonucleosides, or 2'-O-substituted ribonucleoside residues, or any combination thereof. Preferably, such oligonucleotides have from about 8 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The nucleoside residues may be coupled to each other by any of
- 25 the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethyl ester, acetamide,

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carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleotide linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or
5 phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines, and adamantane. The term oligonucleotide also encompasses such polymers as
10 PNA and LNA. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with an -O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl, or allyl group may be unsubstituted or may be substituted, e.g., with
15 halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group.

Particularly preferred antisense oligonucleotides utilized in this
20 aspect of the invention include chimeric oligonucleotides and hybrid oligonucleotides.

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For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or 5 phosphorodithioate region, preferably comprising from about 2 to about 12 nucleotides, and an alkylphosphonate or alkylphosphonothioate region (see e.g., Pederson *et al.* U.S. Patent Nos. 5,635,377 and 5,366,878). Preferably, such chimeric oligonucleotides contain at least three consecutive internucleoside linkages selected from phosphodiester and 10 phosphorothioate linkages, or combinations thereof.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred embodiment of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-O-substituted ribonucleotide region, preferably 15 comprising from about 2 to about 12 2'-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide will contain at least three consecutive deoxyribonucleosides and will also contain ribonucleosides, 2'-O-substituted ribonucleosides, or combinations thereof (see e.g., Metelev and Agrawal, U.S. Patents Nos. 5,652,355 and 20 5,652,356).

The exact nucleotide sequence and chemical structure of an antisense oligonucleotide utilized in the invention can be varied, so long as the oligonucleotide retains its ability to inhibit expression of a specific histone deacetylase isoform or inhibit one or more histone deacetylase 25 isoforms, but less than all specific histone deacetylase isoforms. This is readily determined by testing whether the particular antisense oligonucleotide is active by quantitating the amount of mRNA encoding a specific histone deacetylase isoform, quantitating the amount of histone

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deacetylase isoform protein, quantitating the histone deacetylase isoform enzymatic activity, or quantitating the ability of the histone deacetylase isoform to inhibit cell growth in a *an in vitro or in vivo* cell growth assay, all of which are described in detail in this specification. The term "inhibit expression" and similar terms used herein are intended to encompass any one or more of these parameters.

Antisense oligonucleotides utilized in the invention may conveniently be synthesized on a suitable solid support using well-known chemical approaches, including H-phosphonate chemistry, 10 phosphoramidite chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase oligonucleotide synthesis, such as controlled-pore glass (CPG) (see, *e.g.*, 15 Pon, R. T., Methods in Molec. Biol. 20: 465-496, 1993).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of specific histone deacetylase isoforms by being used to inhibit the activity of specific histone deacetylase isoforms in an 20 experimental cell culture or animal system and to evaluate the effect of inhibiting such specific histone deacetylase isoform activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide that inhibits one or more histone deacetylase isoform expression according to the invention and observing any phenotypic 25 effects. In this use, the antisense oligonucleotides according to the invention is preferable to traditional "gene knockout" approaches because it is easier to use, and can be used to inhibit specific histone deacetylase isoform activity at selected stages of development or differentiation.

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- Preferred antisense oligonucleotides of the invention inhibit either the transcription of a nucleic acid molecule encoding the histone deacetylase isoform, and/or the translation of a nucleic acid molecule encoding the histone deacetylase isoform, and/or lead to the degradation of such nucleic acid. Histone deacetylase-encoding nucleic acids may be RNA or double stranded DNA regions and include, without limitation, intronic sequences, untranslated 5' and 3' regions, intron-exon boundaries as well as coding sequences from a histone deacetylase family member gene. For human sequences, see e.g., Yang et al., *Proc. Natl. Acad. Sci. (USA)* 5 93(23): 12845-12850, 1996; Furukawa et al., *Cytogenet. Cell Genet.* 73(1-2): 130-133, 1996; Yang et al., *J. Biol. Chem.* 272(44): 28001-28007, 1997; Betz et 10 al., *Genomics* 52(2): 245-246, 1998; Taunton et al., *Science* 272(5260): 408-411, 1996; and Dangond et al., *Biochem. Biophys. Res. Commun.* 242(3): 648-652, 1998).
- 15 Particularly preferred non-limiting examples of antisense oligonucleotides of the invention are complementary to regions of RNA or double-stranded DNA encoding a histone deacetylase isoform (e.g., HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8). (see e.g., GenBank Accession No. U50079 for human HDAC-1 20 (Fig. 1B); GenBank Accession No. U31814 for human HDAC-2; (Fig. 2B) GenBank Accession No. U75697 for human HDAC-3 (Fig. 3B; GenBank Accession No. AB006626 for human HDAC-4 (Fig. 4B); GenBank Accession No. AB011172 for human HDAC-5 (Fig. 5B); GenBank Accession No. AJ011972 for human HDAC-6 (Fig. 6B); GenBank Accession No. AF239243 25 for human HDAC-7 (Fig. 7B); and GenBank Accession No. AF230097 for human HDAC-8 (Fig. 8B)).

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The sequences encoding histone deacetylases from many non-human animal species are also known (see, for example, GenBank Accession Numbers X98207 (murine HDAC-1); NM_008229 (murine HDAC-2); NM_010411 (murine HDAC-3); NM_006037 (murine HDAC-4);
5 NM_010412 (murine HDAC-5); NM_010413 (murine HDAC-6); and AF207749 (murine HDAC-7)). Accordingly, the antisense oligonucleotides of the invention may also be complementary to regions of RNA or double-stranded DNA that encode histone deacetylases from non-human animals.
10 Antisense oligonucleotides according to these embodiments are useful as tools in animal models for studying the role of specific histone deacetylase isoforms.

Particularly, preferred oligonucleotides have nucleotide sequences of from about 13 to about 35 nucleotides which include the nucleotide sequences shown in Table I. Yet additional particularly preferred
15 oligonucleotides have nucleotide sequences of from about 15 to about 26 nucleotides of the nucleotide sequences shown below. Most preferably, the oligonucleotides shown below have phosphorothioate backbones, are 20-26 nucleotides in length, and are modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four
20 nucleotides at the 3' end of the oligonucleotide each have 2'-O- methyl groups attached to their sugar residues.

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Antisense oligonucleotides used in the present study are shown in
Table I.

Table 1

5 Sequences of Human Isotype-Specific Antisense (AS)
Oligonucleotides and Their Mismatch (MM) Oligonucleotides

Oligo	Target	Accession Number	Nucleotide Position	Sequence	Gene Position
HDAC1 AS1	Human HDAC1	U50079	1585-1604	5'-GAAACGTGAGGGACTCAGCA-3' (SEQ ID NO:17)	3'-UTR
HDAC1 AS2	Human HDAC1	U50079	1565-1584	5'-GGAAGCCAGAGCTGGAGAGG-3' (SEQ ID NO:18)	3'-UTR
HDAC1 MM	Human HDAC1	U50079	1585-1604	5'-GTTACCTGAGGCCTGAGGA-3' (SEQ ID NO:19)	3'-UTR
HDAC2 AS	Human HDAC2	U31814	1643-1622	5'-GCTGACCTGTTCTGATTTGG-3' (SEQ ID NO:20)	3'-UTR
HDAC2 MM	Human HDAC2	U31814	1643-1622	5'-CGTGACCACTTCTCATTTCC-3' (SEQ ID NO:21)	3'-UTR
HDAC3 AS	Human HDAC3	AF039703	1276-1295	5'-CGCTTCCCTGTCAATTGACA-3' (SEQ ID NO:22)	3'-UTR
HDAC3 MM	Human HDAC3	AF039703	1276-1295	5'-GCCTTCCTACTCATTTGTGT-3' (SEQ ID NO:23)	3'-UTR
HDAC4 AS1	Human HDAC4	AB006626	514-33	5'-GCTGCCCTGCCGTGCCACCC-3' (SEQ ID NO:24)	5'-UTR
HDAC4 MM1	Human HDAC4	AB006626	514-33	5'-CGTGCCCTGCCGTGCCACGG-3' (SEQ ID NO:25)	5'-UTR
HDAC4 AS2	Human HDAC4	AB006626	7710-29	5'-TACAGTCCATGCAACCTCCA-3' (SEQ ID NO:26)	3'-UTR
HDAC4 MM4	Human HDAC4	AB006626	7710-29	5'-ATCAGTCCAACCAACCTCGT-3' (SEQ ID NO:27)	3'-UTR
HDAC5 AS	Human HDAC5	AF039691	2663-2682	5'-CTTCGGTCTCACCTGTTGG-3' (SEQ ID NO:28)	3'-UTR
HDAC6 AS	Human HDAC6	AJ011972	3791-3810	5'-CAGGCTGGAATGAGCTACAG-3' (SEQ ID NO:29)	3'-UTR
HDAC6 MM	Human HDAC6	AJ011972	3791-3810	5'-GACGCTGCAATCAGCTAGAC-3' (SEQ ID NO:30)	3'-UTR
HDAC7 AS	Human HDAC7	AF239243	2896-2915	5'-CTTCAGCCAGGATGCCACCA-3' (SEQ ID NO:31)	3'-UTR
HDAC8 AS1	Human HDAC8	AF230097	51-70	5'-CTCCGGCTCTCCATCTCC-3' (SEQ ID NO:32)	5'-UTR
HDAC8 AS2	Human HDAC8	AF230097	1328-1347	5'-AGCCAGCTGCCACTTGATGC-3' (SEQ ID NO:33)	3'-UTR

The antisense oligonucleotides according to the invention may
optionally be formulated with any of the well known pharmaceutically
10 acceptable carriers or diluents (see preparation of pharmaceutically
acceptable formulations in, e.g., Remington's Pharmaceutical Sciences, 18th
Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990), with the
proviso that such carriers or diluents not affect their ability to modulate
HDAC activity.

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By way of non-limiting example, the agent of the first aspect of the invention may also be a small molecule inhibitor. The term "small molecule" as used in reference to the inhibition of histone deacetylase is used to identify a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably less than 600 Da, which is capable of interacting with a histone deacetylase and inhibiting the expression of a nucleic acid molecule encoding an HDAC isoform or activity of an HDAC protein. Inhibiting histone deacetylase enzymatic activity means reducing the ability of a histone deacetylase to remove an acetyl group from a histone. In some preferred embodiments, such reduction of histone deacetylase activity is at least about 50%, more preferably at least about 75%, and still more preferably at least about 90%. In other preferred embodiments, histone deacetylase activity is reduced by at least 95% and more preferably by at least 99%. In one certain embodiment, the small molecule inhibitor is an inhibitor of one or more but less than all HDAC isoforms. By "all HDAC isoforms" is meant all proteins that specifically remove an epsilon acetyl group from an N-terminal lysine of a histone, and includes, without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8, all of which are considered "related proteins," as used herein.

Most preferably, a histone deacetylase small molecule inhibitor interacts with and reduces the activity of one or more histone deacetylase isoforms (*e.g.*, HDAC-1 and/or HDAC-4), but does not interact with or reduce the activities of all of the other histone deacetylase isoforms (*e.g.*, HDAC-2 and HDAC-6). As discussed below, a preferred histone deacetylase small molecule inhibitor is one that interacts with and reduces the enzymatic activity of a histone deacetylase isoform that is involved in tumorigenesis.

Non-limiting examples of small molecule inhibitors useful for the invention are presented in Table 2.

Table 2

Cpd	Inhibitor Structure	Enzyme IC50 (μM)						% inhibitor of tumor formation in vivo				
		HDAC1	HDAC2	HDAC3	HDAC4	HDAC6	H4-Ac	MTT	Cell Cycle Arrest EC	colon	lung	prostate
1		3	25	21	23	>50	1	3	2			
2		3	31	30	35	>30	5	4	8	53 (40,po)	54 (50,ip)	
3		3	22	45	28	>50	5	4	2	55 (40,ip)		

note: for *in vivo* antitumor studies, numbers outside brackets indicate % of inhibition of tumor growth *in vivo*; numbers in brackets indicate daily dose of inhibitor used (mg/kg body weight/day); oral (PO) or intraperitoneal (IP) administration is indicated in brackets.

5 The reagents according to the invention are useful as analytical tools and as therapeutic tools, including as gene therapy tools. The invention also provides methods and compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects.

10 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell comprising contacting the cell with an agent of the first aspect of the invention. By way of non-limiting example, the agent may be an antisense oligonucleotide or a small molecule inhibitor that inhibits the expression of
15 one or more, but less than all, specific histone deacetylase isoforms in the cell.

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- In one certain embodiment, the invention provides a method comprising contacting a cell with an antisense oligonucleotide that inhibits one or more but less than all histone deacetylase isoforms in the cell. Preferably, cell proliferation is inhibited in the contacted cell. Thus, the
- 5 antisense oligonucleotides according to the invention are useful in therapeutic approaches to human diseases including benign and malignant neoplasms by inhibiting cell proliferation in cells contacted with the antisense oligonucleotides. The phrase "inhibiting cell proliferation" is used to denote an ability of a histone deacetylase antisense oligonucleotide
- 10 or a small molecule histone deacetylase inhibitor (or combination thereof) to retard the growth of cells contacted with the oligonucleotide or small molecule inhibitor, as compared to cells not contacted. Such an assessment of cell proliferation can be made by counting contacted and non-contacted cells using a Coulter Cell Counter (Coulter, Miami, FL) or a
- 15 hemacytometer. Where the cells are in a solid growth (e.g., a solid tumor or organ), such an assessment of cell proliferation can be made by measuring the growth with calipers, and comparing the size of the growth of contacted cells with non-contacted cells. Preferably, the term includes a retardation of cell proliferation that is at least 50% of non-contacted cells.
- 20 More preferably, the term includes a retardation of cell proliferation that is 100% of non-contacted cells (*i.e.*, the contacted cells do not increase in number or size). Most preferably, the term includes a reduction in the number or size of contacted cells, as compared to non-contacted cells. Thus, a histone deacetylase antisense oligonucleotide or a histone
- 25 deacetylase small molecule inhibitor that inhibits cell proliferation in a contacted cell may induce the contacted cell to undergo growth retardation, to undergo growth arrest, to undergo programmed cell death (*i.e.*, to apoptose), or to undergo necrotic cell death.

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- Conversely, the phrase "inducing cell proliferation" and similar terms are used to denote the requirement of the presence or enzymatic activity of a specific histone deacetylase isoform for cell proliferation in a normal (*i.e.*, non-neoplastic) cell. Hence, over-expression of a specific
- 5 histone deacetylase isoform that induces cell proliferation may or may not lead to increased cell proliferation; however, inhibition of a specific histone deacetylase isoform that induces cell proliferation will lead to inhibition of cell proliferation.

- The cell proliferation inhibiting ability of the antisense
- 10 oligonucleotides according to the invention allows the synchronization of a population of a-synchronously growing cells. For example, the antisense oligonucleotides of the invention may be used to arrest a population of non-neoplastic cells grown *in vitro* in the G1 or G2 phase of the cell cycle. Such synchronization allows, for example, the identification of gene
- 15 and/or gene products expressed during the G1 or G2 phase of the cell cycle. Such a synchronization of cultured cells may also be useful for testing the efficacy of a new transfection protocol, where transfection efficiency varies and is dependent upon the particular cell cycle phase of the cell to be transfected. Use of the antisense oligonucleotides of the
- 20 invention allows the synchronization of a population of cells, thereby aiding detection of enhanced transfection efficiency.

- The anti-neoplastic utility of the antisense oligonucleotides according to the invention is described in detail elsewhere in this specification.
- 25 In yet other preferred embodiments, the cell contacted with a histone deacetylase antisense oligonucleotide is also contacted with a histone deacetylase small molecule inhibitor.

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- In a few preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide. As mentioned above, the antisense oligonucleotides according to the invention may optionally be formulated well known
- 5 pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more one or more additional histone deacetylase antisense oligonucleotide(s), and/or one or more histone deacetylase small molecule inhibitor(s), or it may contain any other pharmacologically active agent.
- 10 In a particularly preferred embodiment of the invention, the antisense oligonucleotide is in operable association with a histone deacetylase small molecule inhibitor. The term "operable association" includes any association between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor which allows an antisense
- 15 oligonucleotide to inhibit one or more specific histone deacetylase isoform-encoding nucleic acid expression and allows the histone deacetylase small molecule inhibitor to inhibit specific histone deacetylase isoform enzymatic activity. One or more antisense oligonucleotide of the invention may be operably associated with one or more histone deacetylase small molecule
- 20 inhibitor. In some preferred embodiments, an antisense oligonucleotide of the invention that targets one particular histone deacetylase isoform (e.g., HDAC-1) is operably associated with a histone deacetylase small molecule inhibitor which targets the same histone deacetylase isoform. A preferred operable association is a hydrolyzable. Preferably, the hydrolyzable
- 25 association is a covalent linkage between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor. Preferably, such covalent linkage is hydrolyzable by esterases and/or amidases. Examples of such hydrolyzable associations are well known in the art. Phosphate esters are particularly preferred.

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- In certain preferred embodiments, the covalent linkage may be directly between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor so as to integrate the histone deacetylase small molecule inhibitor into the backbone. Alternatively, the covalent linkage
- 5 may be through an extended structure and may be formed by covalently linking the antisense oligonucleotide to the histone deacetylase small molecule inhibitor through coupling of both the antisense oligonucleotide and the histone deacetylase small molecule inhibitor to a carrier molecule such as a carbohydrate, a peptide or a lipid or a glycolipid. Other
- 10 preferred operable associations include lipophilic association, such as formation of a liposome containing an antisense oligonucleotide and the histone deacetylase small molecule inhibitor covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphatidylcholine, cholesterol,
- 15 phosphatidylethanolamine, and synthetic neoglycolipids, such as syalylacNAc-HDPE. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when the antisense oligonucleotide is associated with one liposome and the small molecule inhibitor is associated
- 20 with another liposome.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the

25 invention. In one certain embodiment, the agent is an antisense oligonucleotide of the first aspect of the invention, and the method further comprises a pharmaceutically acceptable carrier. The antisense oligonucleotide and the pharmaceutically acceptable carrier are administered for a therapeutically effective period of time. Preferably, the

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animal is a mammal, particularly a domesticated mammal. Most preferably, the animal is a human.

The term "neoplastic cell" is used to denote a cell that shows aberrant cell growth. Preferably, the aberrant cell growth of a neoplastic cell is increased cell growth. A neoplastic cell may be a hyperplastic cell, a cell that shows a lack of contact inhibition of growth *in vitro*, a benign tumor cell that is incapable of metastasis *in vivo*, or a cancer cell that is capable of metastases *in vivo* and that may recur after attempted removal. The term "tumorigenesis" is used to denote the induction of cell proliferation that leads to the development of a neoplastic growth.

The terms "therapeutically effective amount" and "therapeutically effective period of time" are used to denote known treatments at dosages and for periods of time effective to reduce neoplastic cell growth. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal, or intrarectal. When administered systemically the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.1 μ M to about 10 μ M. For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. One of skill in the art will appreciate that such therapeutic effect resulting in a lower effective concentration of the histone deacetylase inhibitor may vary considerably depending on the tissue, organ, or the particular animal or patient to be treated according to the invention.

In a preferred embodiment, the therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μ M to about 20 μ M. In a particularly preferred embodiment, the therapeutic composition

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is administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.05 μ M to about 15 μ M. In a more preferred embodiment, the blood level of antisense oligonucleotide is from about 0.1 μ M to about 10 μ M.

- 5 For localized administration, much lower concentrations than this may be therapeutically effective. Preferably, a total dosage of antisense oligonucleotide will range from about 0.1 mg to about 200 mg oligonucleotide per kg body weight per day. In a more preferred embodiment, a total dosage of antisense oligonucleotide will range from
- 10 about 1 mg to about 20 mg oligonucleotide per kg body weight per day. In a most preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 10 mg oligonucleotide per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective amount of a histone deacetylase antisense oligonucleotide is about 5 mg oligonucleotide per kg body weight per day.
- 15

In certain preferred embodiments of the third aspect of the invention, the method further comprises administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time. In some preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide, as described *supra*.

The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μ M to about 10 μ M. In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule

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inhibitor from about $0.05\mu M$ to about $10\mu M$. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about $0.1\mu M$ to about $5\mu M$. For localized administration, much lower concentrations than this may be effective. Preferably, a total
5 dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred
10 embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective synergistic amount of histone deacetylase small molecule inhibitor (when administered with an antisense oligonucleotide)
15 is about 5 mg per kg body weight per day.

Certain preferred embodiments of this aspect of the invention result in an improved inhibitory effect, thereby reducing the therapeutically effective concentrations of either or both of the nucleic acid level inhibitor (*i.e.*, antisense oligonucleotide) and the protein level inhibitor (*i.e.*, histone deacetylase small molecule inhibitor) required to obtain a given inhibitory effect as compared to those necessary when either is used individually.
20

Furthermore, one of skill will appreciate that the therapeutically effective synergistic amount of either the antisense oligonucleotide or the histone deacetylase inhibitor may be lowered or increased by fine tuning
25 and altering the amount of the other component. The invention therefore provides a method to tailor the administration/treatment to the particular exigencies specific to a given animal species or particular patient. Therapeutically effective ranges may be easily determined for example

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empirically by starting at relatively low amounts and by step-wise increments with concurrent evaluation of inhibition.

- In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell
- 5 proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the
- 10 contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus
- 15 inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention,
- 20 the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8.
- 25 In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell

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differentiation comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is an small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time.

In certain embodiments where the agent of the first aspect of the invention is a histone deacetylase small molecule inhibitor, therapeutic compositions of the invention comprising said small molecule inhibitor(s) are administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μ M to about

- 10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05 μM to about 10 μM . In a more preferred embodiment, the blood level of histone
- 5 deacetylase small molecule inhibitor is from about 0.1 μM to about 5 μM . For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total
- 10 dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day.
- 15 In a sixth aspect, the invention provides a method for investigating the role of a particular histone deacetylase isoform in cellular proliferation, including the proliferation of neoplastic cells. In this method, the cell type of interest is contacted with an amount of an antisense oligonucleotide that inhibits the expression of one or more specific histone deacetylase isoform,
- 20 as described for the first aspect according to the invention, resulting in inhibition of expression of the histone deacetylase isoform(s) in the cell. If the contacted cell with inhibited expression of the histone deacetylase isoform(s) also shows an inhibition in cell proliferation, then the histone deacetylase isoform(s) is required for the induction of cell proliferation. In
- 25 this scenario, if the contacted cell is a neoplastic cell, and the contacted neoplastic cell shows an inhibition of cell proliferation, then the histone deacetylase isoform whose expression was inhibited is a histone deacetylase isoform that is required for tumorigenesis. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2,

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HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

- Thus, by identifying a particular histone deacetylase isoform that is required for in the induction of cell proliferation, only that particular histone deacetylase isoform need be targeted with an antisense oligonucleotide to inhibit cell proliferation or induce differentiation. Consequently, a lower therapeutically effective dose of antisense oligonucleotide may be able to effectively inhibit cell proliferation.
- Moreover, undesirable side effects of inhibiting all histone deacetylase isoforms may be avoided by specifically inhibiting the one (or more) histone deacetylase isoform(s) required for inducing cell proliferation.

As previously indicated, the agent of the first aspect includes, but is not limited to, oligonucleotides and small molecule inhibitors that inhibit the activity of one or more, but less than all, HDAC isoforms. The measurement of the enzymatic activity of a histone deacetylase isoform can be achieved using known methodologies. For example, Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) describe the assessment of histone deacetylase enzymatic activity by the detection of acetylated histones in trichostatin A treated cells. Taunton et al. (*Science* 272: 408-411, 1996) similarly describes methods to measure histone deacetylase enzymatic activity using endogenous and recombinant HDAC. Both Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) and Taunton et al. (*Science* 272: 408-411, 1996) are hereby incorporated by reference.

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Preferably, the histone deacetylase small molecule inhibitor(s) of the invention that inhibits a histone deacetylase isoform that is required for induction of cell proliferation is a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of fewer
5 than all histone deacetylase isoforms.

In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase
10 isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6,
15 HDAC-7, or HDAC-8.

The phrase "inducing cell differentiation" and similar terms are used to denote the ability of a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or combination thereof) to induce differentiation in a contacted cell as compared to a cell that is not
20 contacted. Thus, a neoplastic cell, when contacted with a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or both) of the invention, may be induced to differentiate, resulting in the production of a daughter cell that is phylogenetically more advanced than the contacted cell.

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- In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two of the reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a
- 5 histone deacetylase small molecule inhibitor, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain preferred embodiments, each of
- 10 the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.
- Antisense oligonucleotides that inhibit DNA methyltransferase are
- 15 described in Szyf and von Hofe, U.S. Patent No. 5,578,716, the entire contents of which are incorporated by reference. DNA methyltransferase small molecule inhibitors include, without limitation, 5-aza-2'-deoxycytidine (5-aza-dC), 5-fluoro-2'-deoxycytidine, 5-aza-cytidine (5-aza-C), or 5,6-dihydro-5-aza-cytidine.
- 20 In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In preferred embodiments, the cell
- 25 proliferation is neoplasia.

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For purposes of this aspect, it is unimportant how the specific HDAC isoform is inhibited. The present invention has provided the discovery that specific individual HDACs are involved in cell proliferation or differentiation, whereas others are not. As demonstrated in this
5 specification, this is true regardless of how the particular HDAC isoform(s) is/are inhibited.

By the term "modulating" proliferation or differentiation is meant altering by increasing or decreasing the relative amount of proliferation or differentiation when compared to a control cell not contacted with an agent
10 of the first aspect of the invention. Preferably, there is an increase or decrease of about 10% to 100%. More preferably, there is an increase or decrease of about 25% to 100%. Most preferably, there is an increase or decrease of about 50% to 100%. The term "about" is used herein to indicate a variance of as much as 20% over or below the stated numerical values.

15 In certain preferred embodiments, the histone deacetylase isoform is selected from HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1.

The following examples are intended to further illustrate certain
20 preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the
25 appended claims.

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EXAMPLES

Example 1

Synthesis and Identification of Antisense Oligonucleotides

5 Antisense (AS) and mismatch (MM) oligodeoxynucleotides (oligos) were designed to be directed against the 5'- or 3'-untranslated region (UTR) of the targeted gene. Oligos were synthesized with the phosphorothioate backbone and the 4X4 nucleotides 2'-O-methyl modification on an automated synthesizer and purified by preparative reverse-phase HPLC.

10 All oligos used were 20 base pairs in length.

To identify antisense oligodeoxynucleotide (ODN) capable of inhibiting HDAC-1 expression in human cancer cells, eleven phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-1 gene (GenBank Accession No. U50079) were initially screened in T24 cells at 100 nM. Cells were harvested after 24 hours of treatment, and HDAC-1 RNA expression was analyzed by Northern blot analysis. This screen identified HDAC-1 AS1 and AS2 as ODNs with antisense activity to human HDAC-1. HDAC-1 MM oligo was created as a control; compared to the antisense oligo, it has a 6-base mismatch.

Twenty-four phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-2 gene (GenBank Accession No. U31814) were screened as above. HDAC-2 AS was identified as an ODN with antisense activity to human HDAC-2. HDAC-2 MM was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

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- Twenty-one phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-3 gene (GenBank Accession No. AF039703) were screened as above. HDAC-3 AS was identified as an ODN with antisense activity to human HDAC-3.
- 5 MM oligonucleotide was created as a control; compared to the antisense oligonucleotide, it contains a 6-base mismatch.

Seventeen phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-4 gene (GenBank Accession No. AB006626) were screened as above. HDAC-4 AS1 and AS2 were identified as ODNs with antisense activity to human HDAC-4.

10 HDAC-4 MM1 and MM2 oligonucleotides were created as controls; compared to the antisense oligonucleotides, they each contain a 6-base mismatch.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-5 gene (GenBank Accession No. AF039691) were screened as above. HDAC-5 AS was identified as an ODN with antisense activity to human HDAC-5.

15 Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-6 gene (GenBank Accession No. AJ011972) were screened as above. HDAC-6 AS was identified as an ODN with antisense activity to human HDAC-6.

20 HDAC-6 MM oligo was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

25 Eighteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-7 gene (GenBank Accession No. AF239243) were screened as above. HDAC-7 AS was identified as an ODN with antisense activity to human HDAC-7.

- 45 -

Fourteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-8 gene (GenBank Accession No. AF230097) were screened as above. HDAC-8 AS was identified as an ODN with antisense activity to human HDAC-8.

5

Example 2
HDAC AS ODNs Specifically Inhibit Expression at the mRNA Level

In order to determine whether AS ODN treatment reduced HDAC expression at the mRNA level, human A549 cells were treated with 50 nM of antisense (AS) oligonucleotide directed against human HDAC-3 or its corresponding mismatch (MM) oligo for 48 hours, and A549 cells were treated with 50 nM or 100 nM of AS oligonucleotide directed against human HDAC-1, HDAC-2, HDAC-4, HDAC-5, HDAC-6 or HDAC-7 or the appropriate MM oligonucleotide (100 nM) for 24 hours.

Briefly, human A549 and/or T24 human bladder carcinoma cells were seeded in 10 cm tissue culture dishes one day prior to oligonucleotide treatment. The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were grown under the recommended culture conditions. Before the addition of the oligonucleotides, cells were washed with PBS (phosphate buffered saline). Next, lipofectin transfection reagent (GIBCO BRL Mississauga, Ontario, CA), at a concentration of 6.25 µg/ml, was added to serum free OPTIMEM medium (GIBCO BRL, Rockville, MD), which was then added to the cells. The oligonucleotides to be screened were then added directly to the cells (*i.e.*, one oligonucleotide per plate of cells). Mismatched oligonucleotides were used as controls. The same concentration of oligonucleotide (*e.g.*, 50 nM) was used per plate of cells for each oligonucleotide tested.

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Cells were harvested, and total RNAs were analyzed by Northern blot analysis. Briefly, total RNA was extracted using RNeasy miniprep columns (QIAGEN). Ten to twenty µg of total RNA was run on a formaldehyde-containing 1% agarose gel with 0.5 M sodium phosphate (pH 7.0) as the buffer system. RNAs were then transferred to nitrocellulose membranes and hybridized with the indicated radiolabeled DNA probes. Autoradiography was performed using conventional procedures.

Figures 9A-9I present results of experiments conducted with HDAC-1 (Figure 9A), HDAC-2 (Figure 9B), HDAC-6 (Figure 9C), HDAC-3 (Figure 9D),
10 HDAC-4 (Figures 9E and 9F), HDAC-5 (Figure 9G), HDAC-7 (Figure 9H), and HDAC-8 (Figure 9I) AS ODNs.

Treatment of cells with the respective HDAC AS ODN significantly inhibits the expression of the targeted HDAC mRNA in human A549 cells.

15

Example 3
HDAC ODNs Inhibit HDAC Protein Expression

In order to determine whether treatment with HDAC ODNs would inhibit HDAC protein expression, human A549 cancer cells were treated
20 with 50 nM of paired antisense or its mismatch oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 48 hours. ODN treatment conditions were as previously described.

Cells were lysed in buffer containing 1% Triton X- 100, 0.5 % sodium deoxycholate, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, plus protease
25 inhibitors. Total protein was quantified by the protein assay reagent from Bio-Rad (Hercules, CA). 100 ug of total protein was analyzed by SDS-PAGE. Next, total protein was transferred onto a PVDF membrane and probed with various HDAC-specific primary antibodies. Rabbit anti-

- 47 -

- HDAC-1 (H-51), anti-HDAC-2 (H-54) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1:500 dilution. Rabbit anti-HDAC-3 antibody (Sigma, St. Louis, MO) was used at a dilution of 1:1000. Anti-HDAC-4 antibody was prepared as previously described (Wang, S.H. et al., (1999) *Mol. Cell. Biol.* 19:7816-27), and was used at a dilution of 1:1000.
- 5 Anti-HDAC-6 antibody was raised by immunizing rabbits with a GST fusion protein containing a fragment of HDAC-6 protein (amino acid #990 to #1216, GenBank Accession No. AAD29048). Rabbit antiserum was tested and found only to react specifically to the human HDAC-6 isoform.
- 10 HDAC-6 antiserum was used at 1:500 dilution in Western blots to detect HDAC-6 in total cell lysates. Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-
- 15 Pharmacia Biotech., Inc., Piscataway, NJ).

As shown in Figure 10A, the treatment of cells with HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 ODNs for 48 hours specifically inhibits the expression of the respective HDAC isotype protein. Figure 10B presents dose dependent response for the inhibited expression of HDAC-1 protein in cells treated with two HDAC-1 AS ODNs. As predicted, treatment of cells with the respective mismatch (MM) control oligonucleotide does not result in a significant decrease in HDAC-1 protein expression in the treated cells.

20

In order to demonstrate that the level of HDAC protein expression is an important factor in the cancer cell phenotype, experiments were done to determine the level of HDAC isotype expression in normal and cancer cells. Western blot analysis was performed as described above.

25

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The results are presented in Table 3 clearly demonstrate that HDAC-1, HDAC-2, HDAC-3, HDAC-4, and HDAC-6, isotype proteins are overexpressed in cancer cell lines.

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Table 3
**Expression Level of HDAC Isotypes in Human
 Normal and Cancer Cells**

<u>States of Cell</u>	<u>Tissue Type</u>	<u>Cell Designation</u>	<u>HDAC- 1</u>	<u>HDAC- 2</u>	<u>HDAC- 3</u>	<u>HDAC- 4</u>	<u>HDAC- 6</u>
Normal	Breast Epithelial	HMEC	-	+	++	+	+
Normal	Foreskin Fibroblasts	MRHF	-	+	+	++	+
Cancer	Bladder	T24	+++	++	+++	++	+++
Cancer	Lung	A549	++	+++	+++	+++	++
Cancer	Colon	SW48	+++	+++	+++	+++	+++
Cancer	Colon	HCT116	++++	+++	+++	++++	+++
Cancer	Colon	HT29	+++	+++	+++	+++	+++
Cancer	Colon	NCI-H446	++	++++	+++	++++	++
Cancer	Cervix	HeLa	+++	++++	+++	+++	+++
Cancer	Prostate	DU145	+++	+++	+++	++++	+++
Cancer	Breast	MDA-MB- 231	++	+++	+++	+++	++++
Cancer	Breast	MCF-7	+++	+++	+++	++	++
Cancer	Breast	T47D	+++	+++	+++	++	+++
Cancer	Kidney	293T	+++	++++	++++	++	++
Cancer	Leukemia	K562	+++	++++	++++	++++	++++
Cancer	Leukemia	Jurkat T	+++	++	++++	++	++

(-): not detectable; (+): detectable; (++): 2X over (+); (+++): 5X over (+); (++++): 10X over (+)

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Example 4

Effect of HDAC Isotype Specific OSDNs on Cell Growth and Apoptosis

5 In order to determine the effect of HDAC OSDNs on cell growth and cell death through apoptosis, A549 or T24 cells, MDAMB231 cells, and HMEC cells (ATCC, Manassas, VA) were treated with HDAC OSDNs as previously described.

For the apoptosis study, cells were analyzed using the Cell Death
10 Detection ELISA^{Plus} kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's directions. Typically, 10,000 cells were plated in 96-well tissue culture dishes for 2 hours before harvest and lysis. Each sample was analyzed in duplicate. ELISA reading was done using a MR700 plate reader (DYNEX Technology, Ashford, Middlesex, England) at
15 410 nm. The reference was set at 490 nm.

For the cell growth analysis, human cancer or normal cells were treated with 50 nM of paired AS or MM oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 72 hours. Cells were harvested and cell numbers counted by trypan blue exclusion using a
20 hemocytometer. Percentage of inhibition was calculated as (100 - AS cell numbers/control cell numbers)%.

Results of the study are shown in Figures 11-13, and in Table 4 and Table 5. Treatment of human cancer cells by HDAC-4 AS, and to a lesser extent, HDAC 1 AS, induces growth arrest and apoptosis of various human
25 cancer. The corresponding mismatches have no effect. The effects of HDAC-4 AS or HDAC-1 AS on growth inhibition and apoptosis are significantly reduced in human normal cells. In contrast to the effects of HDAC-4 or HDAC-1 AS oligos, treatment with human HDAC-3 and HDAC-6 OSDNs has no effect on cancer cell growth or apoptosis, and

- 51 -

treatment with human HDAC-2 OSDN has a minimal effect on cancer cell growth inhibition. Since T24 cells are p53 null and A549 cells have functional p53 protein, this induction of apoptosis is independent of p53 activity.

5

Table 4
**Effect of HDAC Isotype-Specific OSDNs on Human Normal
 and Cancer Cells Growth Inhibition (AS vs. MM)**

	<u>Cancer Cells</u>	<u>Normal Cells</u>	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	++(+)	+(+)		+/-		+/-
HDAC-2 AS	+(+)	+/-		-		+/-
HDAC-3 AS	-	-		-		-
HDAC-4 AS1	+++	++		++		+/-
HDAC-6 AS	-	-		+/-		-

"-": no inhibition, "+": <50% inhibition, "++": 50-75% inhibition,

10

"+++": >75% inhibition

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Table 5

**Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Apoptosis After 48 Hour Treatment**

5

	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	+	-		
HDAC-2 AS	-	-	-	-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	+	++	-
HDAC-6 AS	-	-	-	-
TSA (100ng/ml)	++	++	++	+

"-": <= 2x fold over non-specific background; "+": 2-3X fold; "++": 3-5X fold;

"+++": 5-8X fold; "++++": 8X fold

Example 5

10 **Inhibition of HDAC Isotypes Induces the Expression of Growth
Regulatory Genes**

In order to understand the mechanism of growth arrest and apoptosis of cancer cells induced by HDAC-1 or HDAC-4 AS treatment,
15 RNase protection assays were used to analyze the mRNA expression of cell growth regulators (*p21* and *GADD45*) and proapoptotic gene *Bax*.

Briefly, human cancer A549 or T24 cells were treated with HDAC isotype-specific antisense oligonucleotides (each 50 nM) for 48 hours. Total RNAs were extracted and RNase protection assays were performed to
20 analyzed the mRNA expression level of *p21* and *GADD45*. As a control, A549 cells were treated by lipofectin with or without TSA (250 ng/ml) treatment for 16 hours. These RNase protection assays were done

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according to the following procedure. Total RNA from cells was prepared using "RNeasy miniprep kit" from QIAGEN following the manufacturer's manual. Labeled probes used in the protection assays were synthesized using "hStress-1 multiple-probe template sets" from Pharmingen (San

- 5 Diego, California, U.S.A.) according to the manufacturer's instructions.
- Protection procedures were performed using "RPA II™ Ribonuclease Protection Assay Kit" from Ambion, (Austin, Tx) following the manufacturer's instructions. Quantitation of the bands from autoradiograms was done by using Cyclone™ Phosphor System (Packard
- 10 Instruments Co. Inc., Meriden, CT). The results are shown in Figures 14, 15 and Table 6.

Table 6

15 **Up-Regulation of p21, GADD45 and Bax After Cell Treatment with Human HDAC Isotype-Specific Antisenses**

	A549			T24		
	p21	GADD45	Bax	p21	GADD45	Bax
HDAC-1	1.7	5.0	0.8	2.4	3.4	0.9
HDAC-2	1.1	1.2	1.0	1.0	1.0	0.9
HDAC-3	0.7	0.9	1.0	0.9	1.0	1.0
HDAC-4	3.1	5.7	2.6	2.8	2.7	1.9
HDAC-6	1.0	1.0	1.0	1.0	0.8	1.1
TSA vs lipofectin	2.8	0.6	0.8			

Values indicate the fold induction of transcription as measured by RNase protection analysis for the respective AS vs. MM HDAC isotype-specific oligos.

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Results of the experiments are presented in Table 6. The inhibition of HDAC-4 in both A549 and T24 cancer cells dramatically up-regulates both p21 and *GADD45* expression. Inhibition of HDAC-1 by antisense oligonucleotides induces p21 expression but more greatly induces *GADD45*

5 expression. Inhibition of HDAC-4, upregulates *Bax* expression in both A549 and T24 cells. The effect of HDAC-4 AS treatment (50 nM, 48 hrs) on p21 induction in A549 cells is comparable to that of TSA (0.3 to 0.8 uM, 16 hrs).

Experiments were also conducted to examine the affect of HDAC
10 antisense oligonucleotides on HDAC protein expression. In A549 cells, treatment with HDAC-4 antisene oligonucleotides results in a dramatic increase in the level of p21 protein (Figure 15).

Example 6

15 Cyclin Gene Expression Is Repressed by HDAC-1 AS Treatment

Human cancer A549 cells were treated with AS1, AS2 or MM oligo directed human HDAC1 for 48 hours. Total cell lysates were harvested and analyzed by Western blot using antibodies against human HDAC1,
20 cyclin B1, cyclin A and actin (all from Santa Cruz Biotechnology, Inc., Santa Cruz, California). AS1 or AS2 both repress expression of cyclin B1 and A. Downregulation of cyclin A and B1 expression by AS1 and AS2 correlates well with their ability to inhibit cancer cell growth. (Figure 16)

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Example 7

Inhibition of Growth in Soft Agar

- 5 1.3 g granulated agar (DIDFCO) was added to 100 ml deionized water and boiled in a microwave to sterilize. The boiled agar was held at 55°C until further use. Iscove's Modified Dulbecco's Medium (GIBCO/BRL), 100x Penicillin-Streptomycin-Glutamine (GIBCO/BRL) and fetal bovine serum (medicorp) were pre-warmed at 37°C. To 50 ml sterile tubes was added 9 ml Iscove's medium, 2 ml fetal bovine serum and 0.2 ml 100x Pen-Strep-Gln. Then 9 ml 55°C 1.3% agar was added to each tube. The tube contents were mixed immediately, avoiding air bubbles, and 2.5 ml of the mixture was poured into each sterile 6 cm petri dish to form a polymerized bottom layer. Dishes with polymerized bottom layers were then put in a CO₂ incubator at 37°C until further use. In 50 ml sterile tubes were prewarmed at 37°C for each 4 cell lines/samples, 20 ml Iscove's medium, 0.4 ml 100x Pen-Strp-Gln and 8 ml fetal bovine serum. Cells were trypsinized and counted by trypan blue staining and 20,000 cells were aliquotted into a sterile 15 ml tube. To the tube was then added DMEM 20 with low glucose (GIBCO/BRL) + 10% fetal bovine serum + Pen-Strep-Gln to a final volume of 1 ml. To the prewarmed 37°C mix in the 50 ml tube was quickly added 8 ml 55°C 1.3% agar, which was then mixed well. Nine ml of this mixture was then aliquotted to each 1 ml cells in the 15 ml tube which is then mixed and 5 ml aliquotted onto the ploymerized bottom layer of the 6 cm culture plates and allowed to polymerize at room temperature. After polymerization, 2.5 ml bottom layer mix was gently added over the cell layer. Plates were wrapped up in foil paper and
- 10
- 15
- 20
- 25

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incubated in a CO₂ incubator at 37°•C for three weeks, at which time colonies in agar are counted. The results are shown in Figure 17.

- These results demonstrate that an antisense oligonucleotide complementary to HDAC-1 inhibits growth of A549 cells in soft agar, but
- 5 antisense oligonucleotides complementary to HDAC-2 or HDAC-6, or mismatch controls, do not.

Example 8

Inhibition of HDAC Isotypes by Small Molecules

10

In order to demonstrate the identification of HDAC small molecule inhibitors, HDAC small molecule inhibitors were screened in histone deacetylase enzyme assays using various human histone deacetylase isotypic enzymes (*i.e.*, HDAC-1, HDAC-3, HDAC-4 and HDAC-6). Cloned
15 recombinant human HDAC-1, HDAC-3 and HDAC-6 enzymes, which were tagged with the Flag epitope (Grozinger, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**:4868-4873 (1999)) in their C-termini, were produced by a baculovirus expression system in insect cells.

Flag-tagged human HDAC-4 enzyme was produced in human
20 embryonic kidney 293 cells after transformation by the calcium phosphate precipitation method. Briefly, 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and antibiotics. Plasmid DNA encoding Flag-tagged human HDAC-4 was precipitated by ethanol and resuspend in sterile water. DNA-calcium
25 precipitates, formed by mixing DNA, calcium choloride and 2XHEPES-buffered saline solution, were left on 293 cells for 12-16 hours. Cells were return to serum-contained DMEM medium and harvested at 48 hour post transfection for purification of Flag-tagged HDAC-4 enzyme.

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HDAC-1 and HDAC-6 were purified on a Q-Sepharose column, followed by an anti-Flag epitope affinity column. The other HDAC isotypes, HDAC-3 and HDAC-4, were purified directly on an anti-Flag affinity column.

- 5 For the deacetylase assay, 20,000 cpm of an [³H]-metabolically-labeled acetylated histone was used as a substrate. Histones were incubated with cloned recombinant human HDAC enzymes at 37°C. For the HDAC-1 assay, the incubation time was 10 minutes, and for the HDAC-3, HDAC-4 and HDAC-6 assays, the incubation time was 2 hours. All assay conditions were pre-determined
10 to be certain that each reaction was linear. Reactions were stopped by adding acetic acid (0.04 M, final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate, and the released [³H]-acetic acid was quantified by liquid scintillation counting. For the inhibition studies, HDAC enzyme was preincubated with test compounds for 30 minutes at 4°C prior to the
15 start of the enzymatic assay. IC₅₀ values for HDAC enzyme inhibitors were identified with dose response curves for each individual compound and, thereby, obtaining a value for the concentration of inhibitor that produced fifty percent of the maximal inhibition.

20

Example 9

Inhibition of HDAC Activity in Whole Cells by Small Molecules

- T24 human bladder cancer cells (ATCC, Manassas, VA) growing in culture were incubated with test compounds for 16 hours. Histones were
25 extracted from the cells by standard procedures (see e.g. Yoshida *et al.*, *supra*) after the culture period. Twenty µg total core histone protein was loaded onto SDS/PAGE and transferred to nitrocellulose membranes, which were then reacted with polyclonal antibody specific for acetylated histone H-4 (Upstate Biotech Inc., Lake Placid, NY). Horse Radish
30 Peroxidase conjugated secondary antibody was used at a dilution of 1:5000

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to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ). After exposure to film, acetylated H-4 signal was quantitated by densitometry.

- 5 The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit histone deacetylation in whole cells.

Example 10

10 **Inhibition of Cancer Cell Growth by HDAC Small Molecule Inhibitors**

Two thousand (2,000) human colon cancer HCT116 cells (ATCC, Manassas, VA) were used in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to quantitatively determine cell proliferation and cytotoxicity. Typically, HCT116 cells were plated into each well of the 96-well tissue culture plate and left overnight to attach to the plate. Compounds at various concentrations were added into the culture media (final DMSO concentration 1%) and incubated for 72 hours. MTT solution (obtained from Sigma as powder) was added and incubated with the cells for 4 hours at 37°C in incubator with 5% CO₂. During the incubation, viable cells convert MTT to a water-insoluble formazan dye. Solubilizing buffer (50% N,N-dimethylformamide, 20% SDS, pH 4.7) was added to cells and incubated for overnight at 37C in incubator with 5% CO₂. Solubilized dye was quantitated by colorimetric reading at 570 nM using a reference of 630 nM. Optical density values were converted to cell number values by comparison to a standard growth curve for each cell line. The concentration test compound that reduces the total cell number to 50% that of the control treatment, *i.e.*, 1% DMSO, is taken as the EC₅₀ value.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can affect cell proliferation.

5

Example 11

Inhibition by Small Molecules of Tumor Growth in a Mouse Model

Female BALB/c nude mice were obtained from Charles River Laboratories (Charles River, NY) and used at age 8-10 weeks. Human prostate tumor cells (DU145, 2×10^6) or human colon cancer cells (HCT116; 2×10^6) or small lung core A549 2×10^6 were injected subcutaneously in the animal's flank and allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times, then approximately 30 mg tumor fragments were implanted subcutaneously through a small surgical incision under general anaesthesia. Small molecule inhibitor administration by intraperitoneal or oral administration was initiated when the tumors reached a volume of 100 mm^3 . For intraperitoneal administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/day) were dissolved in 100% DMSO and administered daily intraperitoneally by injection. For oral administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/day) were dissolved in a solution containing 65% polyethylene glycol 400 (PEG 400 (Sigma-Aldridge, Mississauga, Ontario, CA, Catalogue No. P-3265), 5% ethanol, and 30% water. Tumor volumes were monitored twice weekly up to 20 days. Each experimental group contained at least 6-8 animals. Percentage inhibition was calculated using volume of tumor from vehicle-treated mice as controls.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit the growth of tumor cells *in vivo*.

5

Example 12

Upregulation of p21 Expression and Down regulation of Cyclin Gene Expression Following Treatment with Small Molecule Inhibitor

- Sulfonamide aniline (compound 3, Table 2) is a small molecule
10 HDAC1 specific inhibitor. Human HCT116 cells were treated with escalating doses of compound 3 for 16 hours. Total cell lysates were harvested and expression of p21^{WAF1}, cyclin B1, cyclin A and actin was analyzed by Western blot. Ariti-p21^{WAF1} antibody was purchased from BD Transduction Laboratories (BD Pharmingen Canada, Missasagua, Ontario).
15 Compound 3 clearly upregulates expression of p21^{WAF1} and represses the expression of cyclin A and B1. The expression profile of these cell cycle regulators correlates well with the ability of compound 3 to inhibit HCT116 proliferation in MTT assays (see Table 2),

20

Example 13

Cell Cycle Arrest Induced by HDAC Small Molecule Inhibitors

- Human cancer HCT116 cells were plated at 2X10⁵ per 10-cm dish and were left to attach to the dish overnight in the incubator. Cells were
25 treated with small molecule inhibitors at various concentrations (1 uM and 10 uM, typically, dissolved in DMSO) for 16 hours. Cells were harvested by trypsinization and washed once in 1X PBS (phosphate buffered saline). The cells were resuspended in about 200ul 1X PBS and were fixed by slowly adding 1 ml 70% ethanol at -20° C and were left at least overnight at

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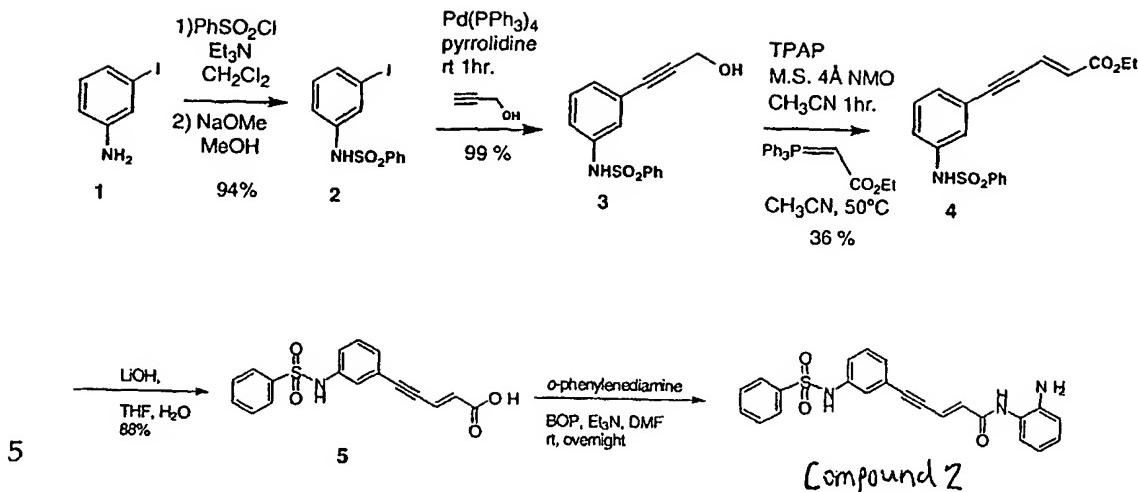
- 20° C. Fixed cells were centrifuged at low speed (1,000 rpm) for 5 minutes, and the cell pellets were washed again with 1X PBS. Nucleic acids from fixed cells were incubated in a staining solution (0.1% (w/v) glucose in 1X PBS containing 50 ug/ml propidium iodide) (Sigma-Aldridge,
5 Mississauga, Ontario, CA) and RNase A (final 100 units/ml, (Sigma-Aldridge, Mississauga, Ontario, CA) for at least 30 minutes in the dark at 25° C. DNA content was measured by using a fluorescence-activated cell sorter (FACS) machine. Treatment of cells with all HDAC small molecule inhibitors in Table 2 results in a significant accumulation of cancer cell in
10 G2/M phase of the cell cycle and concomitantly reduce the accumulation of cancer cells in S phase of the cell cycle. The ratio of cells in G2/M phase vs. cells in the S phase was determined. The Effective concentration (EC) of a small molecule inhibitor to induce a (G2+M)/S ratio of 2.5 is calculated, as shown in Table 2.

15

Example: 14
Synthesis of Small Molecule Compound No. 2

The following provides a synthesis scheme for small molecule Compound No. 2 from Table 2.

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Step 1: 3-(benzenesulfonylamino)-phenyl iodide (2)

To a solution of 3-iodoaniline (5 g, 22.8 mmol), in CH_2Cl_2 (100 mL), 10 were added at room temperature Et_3N (6.97 mL) followed by benzenesulfonyl chloride (5.84 mL). The mixture was stirred 4 h then a white precipitate was formed. A saturated aqueous solution of NaHCO_3 was added and the phases were separated. The aqueous layer was extracted several times with CH_2Cl_2 , and the combined extracts were dried over (MgSO_4) then evaporated. The crude mixture was dissolved in MeOH (100 mL) and NaOMe (6 g), was added and the mixture was heated 1 h at 15 60°C . The solution became clear with time and HCl (1N) was added. The solvent was evaporated under reduced pressure then the aqueous phase was extracted several times with CH_2Cl_2 . The combined organic extracts over (MgSO_4) and evaporated. The crude material was purified by flash chromatography using (100% CH_2Cl_2) as solvent yielding the title 20 compound 21 (7.68g, 94 %) as yellow solid.

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¹H NMR: (300 MHz, CDCl₃): δ 7.82-7.78 (m, 2H), 7.60-7.55 (m, 1H), 7.50-7.42 (m, 4H), 7.10-7.06 (m, 1H), 6.96 (t, J = 8Hz, 1H), 6.87 (broad s, 1H).

Step 2: 3-(benzenesulfonylamino)-phenyl-propargylic alcohol (3)

To a solution of 2 (500 mg, 1.39 mmol) in pyrrolidine (5 mL) at room
5 temperature was added Pd(PPh₃)₄ (80 mg, 0.069 mmol), followed by CuI (26 mg, 0.139 mmol). The mixture was stirred until complete dissolution.
Propargylic alcohol (162 •L, 2.78 mmol) was added and stirred 6 h at room
temperature. Then the solution was treated with a saturated aqueous
solution of NH₄Cl and extracted several times with AcOEt. The combined
10 organic extracts were dried over (MgSO₄) then evaporated. The residue
was purified by flash chromatography using hexane/AcOEt (1:1) as
solvent mixture yielding 3 (395 mg, 99 %) as yellow solid.

¹H NMR: (300 MHz, CDCl₃): δ 7.79-7.76 (m, 2H), 7.55-7.52 (m, 1H), 7.45 (t, J = 8Hz, 2H), 7.19-7.15 (m, 3H), 7.07-7.03 (m, 1H), 4.47 (s, 2H).

15

Step 3: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoate (4)

To a solution of 3 (2.75 g, 9.58 mmol) in CH₃CN (150 mL) at room
temperature were added 4-methylmorpholine N-oxide (NMO, 1.68 g, 14.37
mmol) followed by tetrapropylammonium perruthenate (TPAP, 336 mg,
20 .958 mmol). The mixture was stirred at room temperature 3 h, and then
filtrated through a Celite pad with a fritted glass funnel. To the filtrate
carbethoxymethylenetriphenyl-phosphorane (6.66 g, 19.16 mmol) was
added and the resulting solution was stirred 3 h at room temperature. The
solvent was evaporated and the residue was dissolved in CH₂Cl₂ and
25 washed with a saturated aqueous solution of NH₄Cl. The aqueous layer
was extracted several times with CH₂Cl₂, then the combined organic extract
were dried over (MgSO₄) and evaporated. The crude material was purified

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by flash chromatography using hexane/AcOEt (1:1) as solvent mixture giving 4 (1.21 g, 36%) as yellow oil.

5 ^1H NMR: (300 MHz, CDCl₃): δ 7.81 (d, J = 8 Hz, 2H), 7.56-7.43 (m, 3H), 7.26-7.21 (m, 3H), 7.13-7.11 (m, 1H), 6.93 (d, J = 16 Hz, 1H), 6.29 (d, J = 16 Hz, 1H), 4.24 (q, J = 7 Hz, 2H), 1.31 (t, J = 7 Hz, 3H).

Step 4: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoic acid (5)

To a solution of 4 (888 mg, 2.50 mmol) in a solvent mixture of THF (10 mL) and water (10 mL) at room temperature was added LiOH (1.04 g, 25.01 mmol). The resulting mixture was heated 2 h at 60 °C and treated 10 with HCl (1N) until pH 2. The phases were separated and the aqueous layer was extracted several times with AcOEt. The combined organic extracts were dried over (MgSO₄) then evaporated. The crude residue was purified by flash chromatography using CH₂Cl₂/MeOH (9:1) as solvent mixture yielding 5 (712 mg, 88 %), as white solid.

15 ^1H NMR: (300 MHz, DMSO-d₆): δ 7.78-7.76 (m, 2H), 7.75-7.53 (m, 3H), 7.33-7.27 (m, 1H), 7.19-7.16 (m, 3H), 6.89 (d, J = 16 Hz, 1H), 6.33 (d, J = 16 Hz, 1H).

Step 5: Compound 2

Coupling of 5 with o-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium 20 hexafluorophosphate (BOP) afforded the anilide **Compound 2**.

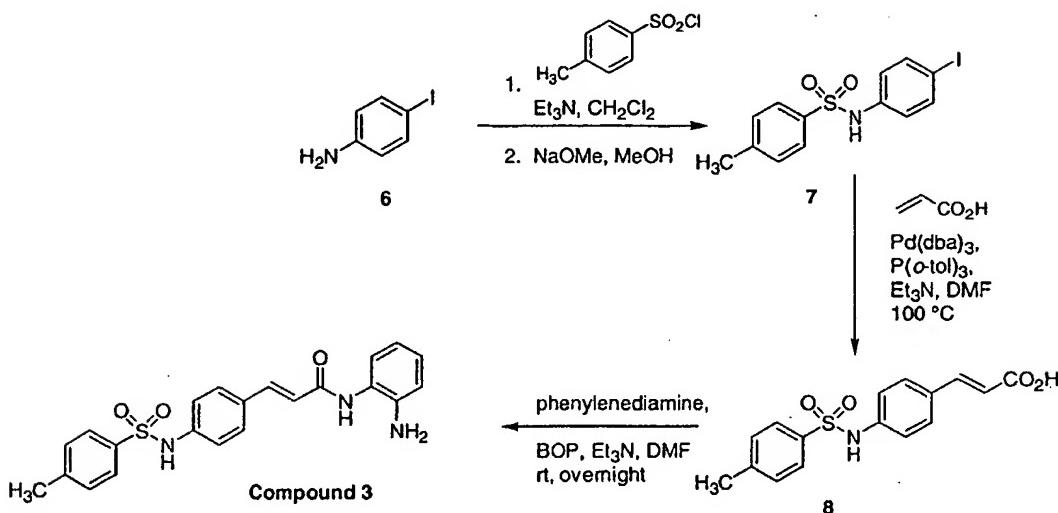
^1H NMR: (300 MHz, DMSO d₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

25 ^{13}C NMR: (75 MHz, DMSO d₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

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Example : 15
Synthesis of Small Molecule Compound No. 3

5 The following provides a synthesis scheme for Compound No. 3 from Table 2.



10 Step 1: 3-[4-(toluenesulfonylamino)-phenyl]-2-propenoic acid (8)

To a solution of 7 (1.39 mmol), in DMF (10 mL) at room temperature were added tris(dibenzylideneacetone)dipalladium(0) ($\text{Pd}(\text{dba})_3$; 1.67 mmol), tri-*o*-tolylphosphine ($\text{P}(o\text{-tol})_3$, 0.83 mmol), Et_3N (3.48 mmol) and finally acrylic acid (1.67 mmol). The resulting solution was degassed and purged several times with N_2 , then heated overnight at 100°C . The solution was filtered through a Celite pad with a fritted glass funnel then the filtrate was evaporated. The residue was purified by flash chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (95:5) as solvent mixture yielding the title compound 8.

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Step 2: N-Hydroxy-3-[4-(benzenesulfonylamino)-phenyl]-2-propenamide

(Compound 3)

The acid 8 was coupled with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium

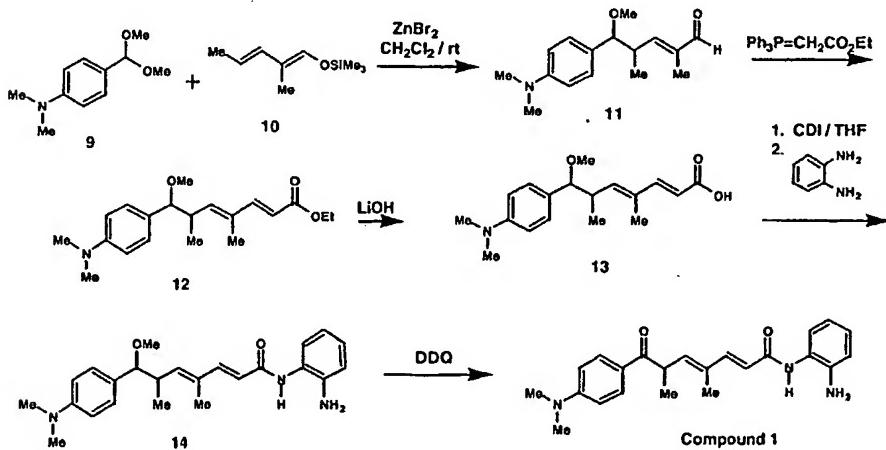
- 5 hexafluorophosphate (BOP) to afford the anilide Compound 3.

^1H NMR: (300 MHz, DMSO d_6): δ 7.77 (broad s, 4H); 7.57 (d, 1H, $J=15.7\text{Hz}$); 7.35 (d, 1H, $J=6.9\text{Hz}$); 7.03-6.94 (m, 6H); 6.76 (d, 1H, $J=7.1\text{ Hz}$); 6.59 (d, 1H, $J=6.9\text{Hz}$); 4.98 (broad s, 2H); 2.19 (s, 3H).

^{13}C NMR: (75 MHz, DMSO d_6): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 16
Synthesis of Small Molecule No. Compound 1

- 15 The following provides a synthesis scheme for small molecule Compound No. 1 from Table 2.



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Step 1: (11)

- To a stirred solution of *p*-anisaldehyde dimethyl acetal (9) (10 mmol) in dry CH₂Cl₂ (60 mL) at rt was added 2-methyl-1-trimethylsilyloxpenta-1,3-diene (10) (*Tetrahedron*, 39: 881 (1983)) (10 mmol) followed by catalytic amount of anhydrous ZnBr₂ (25 mg). After being stirred for 5 h at rt, the reaction was quenched with water (20 mL). The two phases were separated and the aqueous layer was extracted with CH₂Cl₂ (2 × 25 mL). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure.
- Purification of the crude product by flash silica gel chromatography (25% ethyl acetate in hexane) afforded the desired aldehyde 11 in 68% yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ¹H NMR (300 MHz, CDCl₃) δ 9.29 (s, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 6.29 (dq, J = 9.9, 1.2 Hz, 1H), 3.96 (d, J = 6.6 Hz, 1H), 3.20 (s, 3H), 3.05 (m, 1H), 2.94 (s, 6H), 1.60 (d, J = 0.9 Hz, 3H), 1.12 (d, J = 6.9 Hz, 3H).

Step 2: (12)

- A mixture of aldehyde 11 (5.14 mmol) and ethyl (triphenylphosphoranylidene)acetate (2.15 g, 6.16 mmol) in toluene (25 mL) was heated at reflux overnight under N₂. After removal of the solvent under reduced pressure, the crude product obtained was purified by flash silica gel chromatography (10% ethyl acetate in hexane) to give the title compound 12 in 96 % yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ¹H NMR (300 MHz, CDCl₃) δ 7.21 (dd, J = 15.6, 0.9 Hz, 1H), 7.06 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 5.69 (d, J = 15.6 Hz, 1H), 5.67 (br. d, J = 9.0 Hz, 1H), 4.17 (q, J = 7.2 Hz, 2H), 3.87 (d, J = 6.9 Hz, 1H), 3.18 (s, 3H), 2.93 (s, 6H), 2.81 (m, 1H), 1.59 (d, J = 1.2 Hz, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.05 (d, 6.6 Hz, 3H).

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Step 3: (13)

To a stirred solution of diene ester **12** (1.24 mmol) in methanol (10 mL) at rt was added aqueous LiOH 0.5 N solution (1.7mmol). After being stirred at 40 °C for 16 h, methanol was removed under reduced pressure and the resulting aqueous solution was acidified with 3N HCl (pH = ca. 4), extracted with ethyl acetate (25 × 3 mL), dried (MgSO_4), and concentrated under reduced pressure to give the desired carboxylic acid **13** in 98 % yield: **major isomer:** ^1H NMR (300 MHz, CD_3OD) δ 7.21 (d, J = 15.6, 0.6 Hz, 1H), 7.04 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H), 5.61 (d, J = 15.6 Hz, 1H), 5.60 (br. d, J = 10.0 Hz, 1H), 3.85 (d, J = 7.5 Hz, 1H), 3.13 (s, 3H), 2.87 (s, 6H), 2.81 (m, 1H), 1.52 (d, J = 1.5 Hz, 3H), 1.06 (d, J = 6.6 Hz, 3H).

Step 4: (14)

To a solution of carboxylic acid **13** (0.753 mmol) in anhydrous THF (10 mL) was added 1,1'-carbonyldiimidazole (0.790 mmol) at rt, and the mixture was stirred overnight. To the resulting solution was added 1,2-phenylenediamine (5.27 mmol), followed by trifluoroacetic acid (52 μl), and the reaction mixture was stirred for 16 h at rt. The reaction mixture was diluted with ethyl acetate (30 mL), washed with saturated NaHCO_3 solution (5 mL) and then water (10 mL), dried (MgSO_4), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in toluene) afforded the title compound **14** in 61% yield, as a mixture of two isomers in a ca.3 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CD_3OD) δ 7.28-7.02 (m, 5H), 6.79 (m, 2H), 6.68 (d, J = 8.7 Hz, 2H), 5.83 (d, J = 15.0 Hz, 1H), 5.69 (d, J = 9.6 Hz, 1H), 3.87 (d, J = 6.9 Hz, 1H), 3.19 (s, 3H), 2.94 (s, 6H), 2.80 (m, 1H), 1.61 (br. s, 3H), 1.07 (d, J = 6.6 Hz, 3H).

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Step 5: (Compound 1)

To a stirred solution of compound 14 (0.216 mmol) in wet benzene (2 mL, benzene : H₂O = 9 : 1) at room temperature was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.432 mmol). After being stirred
5 vigorously for 15 min., the mixture was diluted with ethyl acetate (30 mL), washed with water (2 × 5 mL), dried (anhydr.MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in hexanes, and then ethyl acetate only) afforded the title compound 35 (6 mg, 7% yield): ¹H NMR (300 MHz, CDCl₃) δ 7.83 (d, J = 9.0, 2H), 7.87 (br. s, 1H),
10 7.29 (d, J = 15.6 Hz, 1H), 7.27 (d, 7.8 Hz, 1H), 7.00 (m, 1H), 6.72 (m, 2H), 6.62 (d, J = 9.0 Hz, 2H), 5.97 (d, J = 15.6 Hz, 1H), 5.97 (d, J = 9.3Hz, 1H), 4.34 (dq, J = 9.3, 6.9 Hz, 1H), 3.03 (s, 3H), 1.87 (br. s, 3H), 1.29 (d, J = 6.9 Hz, 3H); ¹³C
NMR (75 MHz, CDCl₃) δ 12.6, 17.6, 39.9, 40.8, 110.7, 118.0, 119.0, 119.3, 123.8, 124.4, 125.1, 126.9,
15 130.6, 132.5, 140.8, 146.2, 153.4, 164.8, 198.6.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific
20 embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed is:

1. An agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms.
- 5 2. The agent according to claim 1, wherein the agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms, is an oligonucleotide.
- 10 3. The oligonucleotide according to claim 2, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA that encodes a portion of one or more histone deacetylase isoforms.
- 15 4. The oligonucleotide according to claim 3, wherein the oligonucleotide is a chimeric oligonucleotide.
- 20 5. The oligonucleotide according to claim 3, wherein the oligonucleotide is a hybrid oligonucleotide.
- 25 6. The oligonucleotide according to claim 3, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA selected from the group consisting of
 - (a) a nucleic acid molecule encoding a portion of HDAC-1 (SEQ ID NO:2),
 - (b) a nucleic acid molecule encoding a portion of HDAC-2 (SEQ ID NO:4),

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- (c) a nucleic acid molecule encoding a portion of HDAC-3 (SEQ ID NO:6),
- (d) a nucleic acid molecule encoding a portion of HDAC-4 (SEQ ID NO:8),
- 5 (e) a nucleic acid molecule encoding a portion of HDAC-5 (SEQ ID NO:10),
- (f) a nucleic acid molecule encoding a portion of HDAC-6 (SEQ ID NO:12),
- (g) a nucleic acid molecule encoding a portion of HDAC-7 (SEQ 10 ID NO:14), and
- (h) a nucleic acid molecule encoding a portion of HDAC-8 (SEQ ID NO:18).

7. The oligonucleotide according to claim 6 having a nucleotide sequence of from about 13 to about 35 nucleotides.

8. The oligonucleotide according to claim 6 having a nucleotide sequence of from about 15 to about 26 nucleotides.

20 9. The oligonucleotide according to claim 6 having one or more phosphorothioate internucleoside linkage, being 20-26 nucleotides in length, and being modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2'-O- methyl groups attached to their sugar 25 residues.

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10. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-1 (SEQ ID NO:2).

5 11. The oligonucleotide according to claim 10 that is SEQ ID NO:17 or SEQ ID NO:18.

10 12. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-2 (SEQ ID NO:4).

15 13. The oligonucleotide according to claim 12 that is SEQ ID NO:20.

15 14. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-3 (SEQ ID NO:6).

20 15. The oligonucleotide according to claim 14 that is SEQ ID NO:22.

16. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-4 (SEQ ID NO:8).

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24. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-8 (SEQ ID NO:16).

5 25. The oligonucleotide according to claim 24 that is SEQ ID NO:32 or SEQ ID NO:33.

10 26. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the agent according to claim 1.

15 27. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the oligonucleotide according to claim 3.

28. The method according to claim 27, wherein cell proliferation is inhibited in the contacted cell.

29. The method according to claim 27, wherein the
20 oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth retardation.

30. The method according to claim 27, wherein the
25 oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth arrest.

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31. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo programmed cell death.

5 32. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo necrotic cell death.

10 33. The method according to claim 27, further comprising contacting the cell with a histone deacetylase small molecule inhibitor.

15 34. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the agent of claim 1.

20 35. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the oligonucleotide of claim 3.

36. The method according to claim 35, wherein the animal is a human.

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37. The method according to claim 35, further comprising administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time.

5

38. A method for identifying a histone deacetylase isoform that is required for the induction of cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in the induction of cell proliferation indicates that the
10 histone deacetylase isoform is required for the induction of cell proliferation.

39. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

15

40. A method for identifying a histone deacetylase isoform that is required for cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in cell proliferation indicates that the histone deacetylase isoform is required
20 for cell proliferation.

41. The method according to claim 40, wherein the inhibitory agent is an oligonucleotide of claim 3.

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42. A method for identifying a histone deacetylase isoform that is required for the induction of cell differentiation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein an induction of cell differentiation indicates that the histone
5 deacetylase isoform is required for the induction of cell proliferation.

43. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

10 44. A method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a histone deacetylase small molecule inhibitor that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide
15 that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor.

20 45. A method for modulating cell proliferation or differentiation of a cell comprising inhibiting a specific HDAC isoform that is involved in cell proliferation or differentiation by contacting the cell with an agent of claim 1.

46. The method according to claim 45, wherein the cell proliferation is neoplasia.

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47. The method according to claim 46, wherein the histone deacetylase isoform is selected from the group consisting of HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8.

5 48. The method according to claim 47, wherein the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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MAQTQGTRRKVVCYTYDGDVGNYTYGQGHPMKPHRIRMTHNLLLN
YGLYRKMEIYRPHKANAEMTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNNGEDCPV
FDGLFEFCQ1STGGSVASAVKLNKQQTDIAVNWAGGLHHAKKSEASGFCYCYNNDIVLAI
LELIKYHQRVLYIDIDIHHDGVEEAFYTTDRVMTVSFHKYGEYFPFGTGDLRDIGAGK
GKYAAVYPLRDGIDDESYEALFKPVMSKVMEMFQPSAVVIQCGSDSLSGDRRLGCFLNL
TIKGHAKCVEFVKSFNLPMILMLGGGYTIRNCWTYETAVALDTEIPNELPYNDYF
EYFCPDFKLIHISPSNNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPPEE
SGDEDDEDPDKRISICSSDKRIACEEEFSIDSEEEGEGGRKNSSNFKKAKRVKTEDKE
KDPEEKKEVTEEEKTKEEKPEAKGVKEEVKLA (SEQ ID NO:1)

FIG. 1A

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1	atgtctgggg	tctctgcccc	ctggggcccg	tgtctccac	tcggtcatcc	tgagaacaca
61	gcctgtcggr	ctctgtcact	cgggttagac	cacgggtcac	ggcgaggcaag	atggcgaga
121	cgcaggcac	ccggaggaaa	gtctgttact	actacgacgg	ggatgttgg	aattactatt
181	atggacaagg	ccacccaaatg	aaggcttacc	gaatccgcat	ttgtgtctca	ttgtgtctca
241	actatggtt	ctaccgaaaa	atggaaatct	atcgccctca	caaagccaat	gctgaggaga
301	tgaccaagta	ccacaggcat	gactacatta	aatttttgtc	ccagataaca	
361	tgtcgaggta	cagcaaggag	atggcaggat	tcaacgttgg	ccagtattcg	
421	atggcctgtt	ttagttctgt	cagttgtcta	ctgggtgttc	gtgtggaaat	
481	ttaataaggca	gcagacggac	atcgccgtga	attgggtgtga	catggaaac	
541	agtccggggc	atctggcttc	tgttacgtca	atgatatcgt	ctgggaactgc	
601	taaaggatca	ccaggggtg	ctgtacattg	acattgtatat	tcaccatgg	gacggcggtgg
661	aaggggctt	ctacaccacg	gaccgggtca	tgactgtgtc	ctttcataaag	tatggagagt
721	acttcccagg	aacttgggac	ctacgggata	cgggggctgg	caaagacaag	tattatgtctg
781	ttaactaccc	gctccggagac	gggattgtatg	acggatccct	ttcaaggccatt	ttcaaggccgg
841	tcatgtccaa	agtaatggag	atgttccaggc	cstagtgcgg	tgtggctcag	
901	actccctatc	tggggatcgg	ttaggttgct	tcaatctatc	tggtccaaatg	
961	gtgtggaaat	tgtcaaggagg	ttaaccctgc	cstatgtgtat	gctggggggc	
1021	ccattcgtaa	cgttcccgg	tgtctggacat	atgagacagc	tgtggccctg	gatacggaga
1081	tccctaattga	gcttccatac	aatgtactatc	ttgaattactt	tggaccagat	ttcaaggctcc
1141	acatcagtcc	ttccaatatg	actaaccaga	acacgaatga	gtacctggag	aagatcaaac
1201	aggcacttt	tggagaacctt	agaatgtgc	cgcacgcacc	tgggggtccaa	acgcaggcga
1261	ttccctgagga	cggccatccc	gaggaggatgt	ggatgtgagga	cgaaggacgac	cctgacaaggc
1321	gcattctcgat	ctgctccct	gacaaacgaa	ttgcctgtga	ggaaaggatttc	tccgattctcg
1381	aagaggagg	agggggggc	cgcaagaact	ttcccaactt	caaaaaaggcc	aagaggatca
1441	aaacaaaggaa	tggaaaaagg	aaagaccacag	aggagaaggaa	aggagaaggaa	gaagaggaga
1501	aaaccaaggaa	ggagaaaggca	gaaaggcaaaag	gggtcaaggaa	ggggccctcagg	ttggccctgaa
1561	tggaccttc	cagctctggc	ttccctgctga	gtccctcacc	tttctttccc	(SEQ ID NO: 2)

FIG

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MAYSQGGKKCKVCYYYDGDIGNYYYQQGHPMKGPHIRMTHNLLL
NYGLYRKMETIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMHIPIFENVGEDCP
AFDGLFEEFCQLSSTGGSVAGAVKLNQRQTDMAVNWAGGLHHAKKYEASGFCCYVNDIVLA
ILELLKYHQRVLYIDIDIHHRGDGVEEAFYTDVRVMTVSFYGEYFPGTGDLRDIAG
KGKYYAVNFPIMCDGIDDESYGQIFKPIIISKVMMEMYQPSAVVILQCGADSLSGDRLGCFN
LTVKGHAKCVEVVKTFNLPLMI.GGGYTILLRNVARCWTYETAVALDCEIPNIELPYNDY
FEYFGPDEFKLHISPSNMNTNQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHE
DSGDEDGEDPDKRISTRASDKRIACDEEFSDSEDEGGEGGRNTVADHKKGAKARIEED
KKETEDKKTDVKEEDSKDNNSGEKTDTKGTSEQLISNP (SEQ ID NO: 3)

FIG. 2A

1 cggcgagctt tgcggaccc taccgggtgg tcccgaggct
 61 ctccccccggg cctggcccttc cccggggac tatcgcccc
 121 ctctcccccgc cgaggcgggg cggcaggcgg accgtttccct
 181 tggccggcggt gcccattggc tacaggcagg gaggaggagg
 241 tggctactact acgacgggtga tattggaaat tattttatg gaggcgccaa
 301 cctcatagaa tccgcatgac ccataacttg ctgttaattt gacagggtca
 361 gaaatataaa agccccatcaa agccactgcc agtggatgtt
 421 tataatcaaatt ttctacggtc aataagacca gataacatgt
 481 catataatca atgtggaga agattgtccaa gatgttggatg
 541 ctctcaactg gcggttcagt tgctggagct gtgttgcgatg
 601 gctgttaattt gggctggggg attacatcat gcttggatact
 661 tacgttaatg atatttgtct tgccatccctt
 721 tatatggata tagatattca tcatgggtat
 781 cgtgttaatga cggatattcc cccataatatt
 841 aggatatttg gtgtctggaaa aggcaataac
 901 atagacgatg agtcatatgg gcagatattt
 961 tatcaaccctt gttgttgtttt
 1021 ggttgttcca atctaaccat tttttttttt
 1081 acttaccat tactgtatgt
 1141 tggacatatg agactgcat
 1201 gatttttttgg agatttttgg
 1261 aaccggaaaca ctccggaaata tacggaaaaag
 1321 atgtttaccctc atgcacctgg tggccaggatgt
 1381 gacagttggag atgaaggatgg
 1441 aaggggatag ctttgtatga
 1501 agaaatgtgg ctgatcataa
 1561 gaaacagggg acaaaaaaaaac
 1621 gaaaaaacag aaccggatgg
 1681 tctcaccat aaccggaaagg
 1741 gaagacttctt cttttttttt
 1801 actttttcgat
 1861 aaatttctt
 1921 gtcaaaaaaa ctgatcatatt
 1981 aaaag (SEQ ID NO:4)

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MAKTVAYFYDPDVGNFHYGAGHPMKPHRLALTHSLVLYGLYKK
MIVFKPYQASQHDMCRFHSEDYIDFLQRVSPTNMQGFTIKSLNAPNVGDDCPVFPGLFE
FCSRXITGASLQGATQLNNKICDIANWAGGLHHAKKFEASGFCCYYNDIVIGILELLKY
HPRVLYIDIDIHGDGVQEAFYLTDRVMTVSEFHKGNYFFPGTGDMDYEVGAESGRYYC
LNVPLRDGIDDQSYSYKHLFQPVINQVVDFYQOPTCTVLQCGADSLGCDFNLNSIRGH
CECVYRKSFNIPPLLVGGGYTVRNVARCWTYETSLLVEEAISEELPYSEYFYFAP
DFTLHPDVSTRIENQSRQYLDQIRQTIFENLKMLNHAHSVQIHDPADLLTYDRTDE
ADAEEERGPEENYSRPEAPNEFYDGDHDNDKESDVEI (SEQ ID NO: 5)

FIG. 3A

1	ggaaatttcgcg	gcgggggggg	tgccatggcg	gttccccccct
61	caagaccggtg	acggacccttct	cgtggggcga	ttccactacg
121	ccctatgtaa	cccccattggcc	tggcattgtac	gtccctgcatt
181	taagaaggatg	atcgccctca	ggccctccaa	aggctctcta
241	ctccggggac	tacattgact	tccctggagg	gcgcgttcca
301	caaggatctt	aatggcttca	agttagggca	agggttccac
361	gttctgtcg	cgttacacag	tgactggccca	gtgttcccg
421	cgttgcattt	gccattaact	ggccatctct	ggcttccgtga
481	tggcttcgtc	tatgtcaacg	tctggcaccat	accaggctga
541	tcgggtgtctc	tacattgaca	tggcatccat	gccaaggaaat
601	cctcaactgac	cgggtcatga	ccatgggtac	accaggatca
661	cacagggtgac	atgtatggaa	ccacaaatac	gttccctgg
721	cctgggggat	ggcattgtatg	gaggggccgc	tactactgtc
781	ggtaggtggac	ttcttaccaa	caaggacacct	ttccaggccc
841	cgttgatcga	ttgggtctgt	ccacgtgtcat	actctctgg
901	tgtcaaggaggc	ttcaaatatcc	ttaaccctcag	gtgttgcata
961	tgttgcccgcc	tgctggacat	ctctactcgt	gttcccgaaa
1021	gtttccctat	agtgaatact	atggacatc	gaaggaggca
1081	cagccccggc	atcgagaaatc	tgaggtactt	tttagtgagg
1141	ctttgaaaaac	ctgaaggatgc	agaactcact	atccagatgt
1201	agaccttgg	acctacgaca	tgaaaccatgc	gccagacaaat
1261	gaactataggc	aggccagggg	ccagttgttc	acgtggcctgc
1321	ggaaaggcgat	gtggaggattt	catccaaatga	ttgttgcggg
1381	cacctttgg	aagggttgg	gttccatgt	gttccatgt
1441	gggcttttg	ctgactctgg	ggaggatct	gttccatgt
1501	cctgctttc	ctctctctcc	ggggccatca	gttccatgt
1561	caaggataggc	tatctggac	attattggca	gtggggccctg
1621	ttggccctta	tttctccct	gttccctcg	gtggccatcc
1681	agacaaggac	tctgacttcc	tttgcggatc	gttccatgt
1741	ccttgcttcc	aggaaatccca	tgagattggcc	tttttgcggat
1801	ctgaaatccca	gatggatggaa	aaggagaggaa	tttttgcggat
1861	ccttcacttt	atggctttatg	agtagttttt	tttttgcggat
1921	attttttta	tttttttggta	caactttttt	aaatgttctg

FIG. 3B

ggcc (SEQ ID NO: 6)

MILAMKHQQELLEHQRKLERHRQEQELEKQHREQQLQQQLKNKEKG
KESAVASTEVVKMQLQEFVLINKKALAHPPNLIHCISSSCPRYWYGGKTQHSSSLDQSSPPQS
GVSTSYNHPVLGMYDAKDDFPLRKTASEPNLKRSRLKQKVAAERRSSPLLRRKDGPVV
TALKKRPLDVTDSSACSSAPGSGPSSPNNSSGSVSAENGIAPAVPSIAPETSLAHLVVA
REGSAAPLPLYTSPSLPNITLGLPATGPSAGTAGQQDTERLTLPALQQRQLSLFPGTHL
TPYLSTSPLERDGGAHSPLLQHMVLLEQPPAQAPLVTGLGALPLHAQSLLVGADRVSPT
SHIKLRLQRPLRGRTQSAPLPQNAAQALQHLVIQQHQQQFQQQQLQMNNKLIIP
KPSEPARQPESHPEETEEELREHQALLDEPYLDRLPGQKEAHAQAGVQVQKQEPIESDE
FEAEPPREVEPGQRQPSSEQELFRQQALLLEQQRIRHQLRNYQASMEAAGIPVSFGGHR
PLSRAQSSPASATFPVSVQEPPTKPRFTTGLVYDTLMILKHQCTCGSSSSHPEHAGRIQ
SIWSRLLQETGLRGKCECIRGRKATLEELQTVHSEARHTLLYGTNPLNRQKLDSKKLGS
LASVVFVERLPCCGGVGVDSDTIWNEVHSAGAARLAVGCVVELVFKVATGELKNGFAVVRP
PGHAAEESTPMGFCYFNNSVAVAAKLLQQRQLSVSKILIVDWDVHHGNGTQQAFYSDPSV
LYMSLHRYDDGNFFPGSGAPDEVGTGPGVGFNVNMAFTGGLDPPMGDAEYLAAFRTVV
MPIASEFAPDVVLASSGFDAVEGHPTPLGGYNLSARCFGYLTQQLMGLAGGRIVLALE
GGHDLTAICDASEACVSSLGNELDPLPERKVLQQRPNANAVERSMEKVMEIHSKYWRCL
QRTTSTAGRSLIEAQTCENEAEVTAMASLSVGVKPAEKRPDEEPMEEPPL (SEQ ID NO: 7)

FIG. 4A

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1	ggagggttgt	ggccgcgcgc	cgcggggcac	cgtccccccc	cccgagcccgag	cccgagcccgcc
61	agcccgcca	ccgcggccgc	ccgcccgcgc	cgccccccga	acaggctccc	aggctggggcc
121	ccggggggcg	ccgtggccgc	gtcccggttg	tgcggccccc	cgcgcgcgg	cgccctgggg
181	cgggtgggg	cgcagggtga	ggagatgggg	cgggggggcc	cggaggaggg	ctagaggccgg
241	ccggccggcc	cggccgggtt	aaggccggcc	cgccccgggg	gccccggggc	cattgttcgg
301	cgcccccc	ggcccccgg	caggctggag	gccttggagg	cgcgggcagg	tggacggccgc
361	cggccacac	ccggccggcg	cggggccgtg	ggaggggggg	gccagggttg	gccggcgccgc
421	gtgggaccgg	cgggtcccc	ggggccggcg	ggcccccgtt	gaccctttcca	cccggggccgc
491	gagggggtt	cgcggccgg	ggggggggcg	cgggggtggg	cacggcaggc	agcggggccgg
541	tctccgggt	cgggggccgg	gccccccgg	caggttcatc	tgcagaaggc	agcggacggcc
601	tctgttcaac	ttgtgggtta	cctggctcat	gagaccccttg	cggcgaggct	cggcggttgg
661	acgtctgtga	cccaggcctc	accgtcccg	tacttgtatg	tgttgtgggg	agtttggaggc
721	tcgttgtggc	tatcgttttcc	gtggaaattt	tgagccattt	cgaatcaactt	aaaggagggtgg
781	acatttgctag	caatggagtc	ccaaaggccat	ccagatggac	tttctggccc	agaccaggcca
841	gtggaggctgc	tgaatccggc	cggcggtgaac	cacatggcca	gcacgggtgga	tgtggccacg
901	gcgcgtggctc	tgcgaagtggc	ccccccggca	geggccatgg	accggggcct	ggaccaccagg
961	ttctcaactgc	ctgtggcaga	ggccggcccttg	cggggggaggc	aggctgcaggca	ggaggctccctg

FIG. 4B-1

1021	gcgctcaagg	agaaggcaga	gttccagggg	cagatctca	tgcggagg	ccagaggcag
1081	cacggcggc	tctccggca	gcacggggc	cagtcacacg	gcaataacaa	gcaataacag
1141	gagatgtgg	ccatgaaggc	cgaggaggag	ctgtctggaa	accaggcgaa	gttgaggagg
1201	cacggcgg	agcaggaggct	ggagaaggcag	caccggaggc	agaaggctgca	gcaggctcaag
1261	aacaaggaga	aggccaaggaa	gatggccgtg	ggggaggacag	aatgtggaaat	gaagttacaa
1321	gaatttgtcc	tcaaataaaaa	gaaggccgtg	gccccacggg	atctgaacca	ctgacttcc
1381	aggaccctc	gttactggta	cgggaaaacg	cggcaggactt	cccttggacca	gagtttccaa
1441	cccaggcg	gagtgtgac	ctccataac	caccgggtcc	tgggaaatgtt	cgacggccaa
1501	gatgacttcc	ctcttaggaa	aacagttct	gaaccggaaatc	tggaaatcacg	gtccaggctaa
1561	aaggcggaaag	tggccggaaag	acggaggaggc	cccctgtttac	ggggccagggt	ggggccaggta
SUBS 681	gtcactgttc	taaaaaaggcg	tccgttggat	gtcacagact	ccgggtggcag	caggccccaa
HT 741	ggctccggac	ccagctcacc	aaacaacaggc	tccggggagg	tcgcgttggag	gaacgggtatc
801	ggccccggcg	tccccaggcat	cccggggagg	acgggtttgg	cgcacagact	tgtggcacgaa
861	gggctggctg	ccacccggcc	tccctctac	acatcgccat	catacaggtcg	cgaggactc
921	acccttcccg	ccctccaggca	gaggctctcc	cttttccccg	gcaccacact	cactccctac
981	ctgaggacct	cgcccccttgg	ggggggacggg	ggggggaggcg	tctgcaggcac	tctgcaggct
2041	atggtcttac	tggaggcggcc	accggccacaa	gttgggttggca	ggggaggactcg	ggggaggactcg
R101	ccctccacg	cacagtctt	gggtgggtgt	cccccctccat	ccacaaggctg	ccacaaggct
2161	cggcggacc	ggcccaactggg	ggggaccggag	tggggcccg	tggggcccg	cgcccgaggct
2221	ctggcggacc	tggtcatcca	cgaggaggat	caggattttc	tggggaaacaa	caaggcaggag
2281	ttccaggaggc	agcaactggca	gttggaaacaa	atcatcccc	agccaaaggcg	gccaggcccc
2341	caggcggaga	gcacccggaa	gggggggggg	gtggggctcc	gttggggacca	ggctctgtcg
2401	gacggggccct	acttggaccg	gctgggggggg	cagaaggagg	cgcacggcaca	ggccggggcggt
2461	caggtaaggc	aggaggccat	tggaggcgat	gagggaggagg	caggggccccc	acgggggggtg
2521	gaggcggcc	aggccggcc	caggccggcc	gaggctgtct	tcaaggccaa	agccctccctg
2581	ctggaggcgg	aggggatcca	ccaggctgggg	gatgggggg	cgtccatggg	ggccggccggc
2641	atccccgtgt	ccttcgggg	ccacaggccct	ctgtccgggg	cggcgttcc	acccgggtct
2701	gccacccccc	cggttccgtt	ggggggggcc	cccaccaagg	cgggtttcac	gacaggccctc
2761	gtgtatgaca	cgtgtatgtt	ggggccaccagg	tggcaccctgg	gggggttaggg	cagccacccccc
2821	gaggcacggcg	ggggatccca	ggggggatccca	ggggcatctgg	tcccgccgtgc	cctccggggcc

FIG. 4B-2

2881 aaatggcgagg gcatccgggg acgcaaggcc accctggaaag agtacagac ggtgcactcg
 2941 gaaggccaca ccctcctgta tggcacgaac cccctcaacc ggcagaact ggacagtaaag
 3001 aaacttctag gctcgctcgc ctccgtttc gtcgggcttc ttgcggccct tggtgggg
 3061 gacagtgaca ccatatggaa cgagggtgcac tcggcgggcaggccccct ggctgtgggg
 3121 tgcgtgttag agctggtctt caagggtggcc tgcggaggc accggggagg aggaaaaatgg ctgtgttg
 3181 gtccggccccc tgggacacca acgtgggggg tggcaggcca tggaaacccca tgggttttg ctacttcaac
 3241 tccggggccg gtggactggg acgtgtccaa tggcaggccaa gcttctgcag cggctacg gatggaaact
 3301 gtccctgtaca tggcccttca cggctacgac gatggaaact tcttccagg cagggggtt
 3361 gtccctgtaca tggcccttca cggctacgac gatggaaact tcttccagg cagggggtt
 3421 cttgtatggg tggggcagggg gccccgggg gtttcaacg tcaacacggc tttcaccggc
 3481 ggccctggacc ccccatggg agacgctgag tacttgggg ccttcagaac ggtgttaatg
 5541 ccgatcgcca gcgaggttgc cccggatgtt gtgtgtt cataggctt cgtatggcg
 601 gagggccacc ccaccctct tggggctac aacctctcc cagatgtt cgggtacctg
 661 acgaaggcgc tggatggccct ggctgggggg cggattgtcc tggcccttcga
 721 gagctgaccg ccatttgcga cgcctcggaa gcatgttgtt ctggcttgc
 781 cttgtatcc tcccaggaaaa gtttttacag caaagacca atgaaaacgc
 841 atggagaaag tcatggagat ccacagcaag tactgggtt cttccgttcc
 901 acagggggg gttctctgtat cggggctcag acttgcgaga acggaaaggc
 961 accggccatgg cctcgctgtc cgtggacgtt aagccggcg aaaagagacc
 1021 cccatggaaag agggggccgc cctgttagcac tccctcgaag ctgtgttct
 2081 tggctctgtc gggctctttt gggacacca gccaagaaac gccacggaa
 4141 gggctctttt gggacacca gggacacca gggacacca ggtgtcaaca
 4201 cgcccaaggcc cacggctc tggacggcaca tgccacggctg ggcgtggcag
 4261 aacacgggac agacggggcc gacggcaga cacacggaca cggggaaaggc
 4321 tgggggtcc cggaggac gccgtggaaag aaaggagcct gtggcaac
 4381 tgccgaattt agttgacacg aggacacgaa aacaatac aactccac
 4441 caaacttgc taaaactggt gttttttttt ttttttaag aggacgttt
 4501 aaccatcga ctcatctttt agtttattttt tttttttttt
 4561 ggccggccctc tggaaaccat aggggtgtt ggggggggt ctgcacccgg
 4621 gagggacccct taaaaaaacaaaactggac agaaacaggaa atgtgagctg
 4681 cttgggtttc tcggaaaggca tcggaaaggatg cgggttttttattgtctgtt
 FIG. 4B-3

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SUBSTITUTE SHEET (RULE 26)

FIG. 4B-5

6601 acggttcttgg actttccctt ccctgggtt cactttggc tctgaaggccc
 6661 agatggccaa gaggagctgg tccattcccc attcatggca cagaacagtg gagggggcca
 6721 gcttagcaggc tccttcggcc tccttggccctt cattctgc ataggccctt gggatccg
 6781 ccacctggcc tcttaccccg cggggggctta tggggggaa tgcatcactt cactttttt
 6841 tttaaaggcag atgatggat aacatggact gtcaggggc caggttatca gtggggggac
 6901 tttaattctaa tctcattcaa atggagacga cctctgcaa cgcctggcagg ggctggccaa
 6961 gtttcatctg tcagctcact gcgtctgag gggttttttccat aaatgtgtg agggcattac
 7021 ttcttttgaa gacacactcg gggggaccac gtcagggggg tccaggggcag atggcagggg
 7081 atctggctcg tggaccttta tggacttta aaggcaagg tcccttttcat gtgttctccc
 7141 tgggggttcc tggacttta aaggcaggcc ttttatcccc ttcttttgcgggggggggggggg
 7201 gggagtccgc aaggcaggac tctgtggcg cctcggcgct tccggccgtt cggcaggcgtt
 7261 cctcttggcg tctgaccctgc ctcgggggg ctggccgggggggggggggggggggggggg
 7321 cacccccac cccaggcgcc tgggggtctt ggttctgggggggggggggggggggggggg
 7381 cccaggcgcc tggggccatctt ttgaaaatctt ttggcaagg gggaggaccatccatcc
 7441 ttacttctt ttgaaaatctt ttgaaaatctt ttggcaagg gggaggaccatccatcc
 7501 aaagcaagg ttgattttgc agcacccatggc atggacttgc ttgttttttttttttttttttt
 7561 aacattccctt ctttacttggt caccggccatggc ttgttttttttttttttttttttttt
 7621 tggggccacg tggatcatttt gcatggatac atatataat atatagatat aaatataat
 7681 gaaatcatttt ttttaatgg tatacaggatt tggcacccca aactcggcagg ggactgtacg
 7741 ctttatattg tggatcatttt tggatcatttt tggatcatttt tggatcatttt tggatcatttt
 7801 gcctttctgt tggatcatttt tggatcatttt tggatcatttt tggatcatttt tggatcatttt
 7861 acaaaaaccc ttggatcatttt tggatcatttt tggatcatttt tggatcatttt tggatcatttt
 7921 aaattgtact ttggatcatttt tggatcatttt tggatcatttt tggatcatttt tggatcatttt
 7981 gaaaaaaagg ttatcttagca ttggggggaa ctggggggaa ttggatcatttt tggatcatttt
 8041 gaaaacagggt ttggggggaa ctggggggaa ttggatcatttt tggatcatttt tggatcatttt
 8101 tgatttggag gaattttgtt ttggatcatttt tggatcatttt tggatcatttt tggatcatttt
 8161 gccaggcgag cgccggccggc ctttactggc ttgttgacgg ttggatcatttt tggatcatttt
 8221 gggggactcg aaaggatccc ttggggggaa ttggatcatttt tggatcatttt tggatcatttt
 8281 gtgatgtatg gctaaggatttt ctttactggc ttggatcatttt tggatcatttt tggatcatttt
 8341 caattatact ttggatcatggaa agggaaaccat ttggatcatttt tggatcatttt tggatcatttt
 8401 ttagctcgcc cttcaactttgtt ttggatcatttt tggatcatttt tggatcatttt tggatcatttt

SUBSTITUTE SHEET (RULE 26)

aagaaaaagg (SEQ ID NO: 8)

LRQGGTILTGKFMSTSSIPGCILLGVALEGDGSPHIGHASHASLLQHVVLL
LEQARQQSTLIAVPLHGQSPLITGERVATSMRTVGKLPRHRPLSRTQSSPLPQSPQAL
QQLVMMQQHQQFLEKQKQQQLQLGKILTKTGEELPROPTTHPEETEEELTEQQEVLLGE
GALTMPREGSTESESTQEDLEEEDEEEDGEEDCIQVKDEEGESGAEEGPDLIEEPGA
GYKKLFDQPLQPLQVYQAFLSLATVPHQALGRTQSSPAAPGGMKSPPDQPVKHLFT
TGVVVYDTFMLKHQCMCGNTHVHPEHAGRIQSIIWSRLQETGLIISKCERIRGRKATLDEI
QTVHSEXIHTLLYGTISPLNRQKLDISKLLGPTISQKMYAVLPCCGGIGVDSDTVWNEMHSS
SAVRMAGCLLEAFKVAAGELKNGFAIRRPGHAAESTAMGFCEFFNSVAITAKLLQ
QKLNVGKVLIIVDWDIHHGNGTQQAFYNDPSVLYISLHYDNGNFPFGSGAPEEVGGGP
GVGYNNVVAWTGGVDPPIGDVYLTAFRFTVVMPIAHEFSPDVVTLVSAAGFDAVEGHLS
LGGSVVTARCFCGHILTRQMLTLAGGRVVLALLEGGHDLTACDAEACVSALLSVELQPL
DELVQQKPNINAVATLEKVIETQSKHWSCVQKFAAGLGRSLREAQAGETEEAETVSA
MALLSVGAEQAAAREHSRPAEEPMEQEPAL (SEQ ID NO: 9)

FIG. 5A

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1	ccctggcca	gggtggcacg	ctgaccggca	atccatcgat	cacatcttct	attcctggct
61	gcctgtgg	cgtggactg	gaggccgacg	ggagccccca	cggccatgcc	tccctgtctgc
121	aggatgtgc	gttgtctggag	caggccggcc	aggcaggacac	cctcattgtct	gtggccactcc
181	acgggcagt	cccacttagtg	acgggttgaac	gttgtggccac	cagcatcgccg	acggtagggca
241	agttcccg	gcattcgccc	ctgagccgca	ctcagtctc	accgcgtccg	cagagtcccc
301	aggccctgca	gcaggctggtc	atgcaacaac	agcaccaggca	gttcctggag	aaggcagaagg
361	agcaaggact	acagctgggc	aagatcctca	ccaaaggacagg	ggaggtcccc	aggcagccca
421	ccaccaccc	tgaggagaca	gaggaggaggc	tgacggggca	gcaggaggtc	ttgctgggg
481	aggaggccct	gaccatgccc	cggggggct	ccacaggagg	ttagaggaca	caggaaggacc
541	tggaggagg	ggacgcggaa	gaggatgggg	aggaggaggaa	ggatttgcac	caggttaagg
601	acggagggg	cgaggatgtgt	gcttgggggg	ggcccgactt	ggggggccct	ggtgctggat
661	aaaaaaact	gttctcagat	gcccaggccgc	tgcaggcttt	gcagggttac	caggcgcccc
721	tcaagcctgg	cacttgtccc	caccaggccc	tggggccgtac	ccagtcctcc	cctgctgccc
781	ctggggcat	gaaggagccc	ccagaccaggc	ccgtcaagca	cctcttacc	acagggttgt
841	tctacgacac	gttcatgtcta	aaggcaccagg	gcatgtggcg	gaacacacac	gtggcaccctg

FIG. 5B-1

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901 agcatgctgg ccggatccag agcatctggg cccggctgca ggagacaggc ctgccttagca
 961 agtggaggcg gatccgagggt cgcaaaaggca cgcttagatga gatccagaca gtgcactctg
 1021 aataccacac cctgctctac gggaccaggc ccctcaaccg gcagaaggcta gacaggcaaga
 1081 agttgctcgcc ccccatcagg cagaagatgt atgttgtgtt gcttgtgggg ggcatcgggg
 1141 tggacagtga caccgtgtgg aatgaggatgc actccctcag tgctgtgtgt atggcagtgg
 1201 gctggctgtct ggaggctggcc ttcaagggtgg ctgcaggaga gctcaagaat ggatttgcca
 1261 tcataccggcc cccaggacac cacggcggagg aatccacagg aatccacagg cacggatcc tgccttcata
 1321 actctgttagc catcacggca aaactcctac aggagaaggta gaacgtggc aagggtccata
 1381 tcgtggactg ggacattcac catggcaatgc gcaaccaggca ggcgttctat aatgaccctt
 1441 ctgtgtctta catctctcg catgctatgc acaacggaaa ttctttcca ggcgtctgggg
 1501 ctcccggaaa ggtttgtggaa ggaccaggcg tgggttacaa tgtgaacgtg gcatggacag
 1561 gaggttgtgg cccccccatt ggagacgtgg agtaccttac agccttcagg acagtggtag
 1621 tgcccattgc ccacgagtttc tcacctgtgg tgggttcttagt ctcggccggg ttgtgtctg
 1681 ttgaaggaca tctgtctccct ctgggtggct actctgtcac cgcccagatgt ttggccact
 1741 tgaccggca gctgtgtgacc ctggcagggg gcccgggtgg gctggccctg gagggaggccc
 1801 atgacttgtac cgcccatctgt gatggcttgc ggagtcttgc agaaaaaggcc caacatcaa
 1861 agctggcggcc ctggatgag ggatgttgc gcaatcgatggc aacactggag ctgtgtcgag
 1921 cgcttagagaa agtcatcgag atccaggaga aacactggag ctgtgtcgag aagttcggcc
 1981 ctggtttgggg cgggtccctg cgaggaggccc aaggaggta ggcggaggag ggcggaggactg
 2041 tgaggccat ggccttgcgt tcgggtgggg ccggaggaggcc cgggtggcc
 2101 aacacagccc caggccggca gaggaggccca tggaggcaggaa gctggccctg tgacgccccgg
 2161 gccccatcc ctctggctt caccattgtg attttgttta ttttttctat taaaaaaaa
 2221 aagtccacaca ttc (SEQ ID NO: 10)

FIG. 5B-2

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1 mtstgqdstt trqrrsrqnp qsppqdsstt skrnikkav prsipnlaev kkkglmkklg
 61 gameedlivg lggmdlnlea ealagtglvl deglnefhcl wddsfpege rhaikedli
 121 qeglldrcvs fgarfaekee lmlvhsleyi dlmettqymn egelrvladt ydsvy1hpns
 181 yscaclasgs virlv dav1g aeirngmai i rppghhahs lmdgrycmfnh vavaaryaqg
 241 khriirvlliv dwdvhhgqt qftfdqdpssv lyfshryeq grfwphlkas nwsttggfqg
 301 qgytinvpwn qvgmrdayi aaflhvllpv alefqpqlvl vaagfdalqg dpkgemaatp
 361 agfaqlthll mglaggkll sleggynira laegvsash tllgdpcpm espgapcrsa
 421 qasvsclea lepfwevlvr stetverdm eednveese egpweppvlp iltwpv1qsr
 481 tg1vydqnum nhcnlwds hh pевpqrilri morleelgia grcltitprp ateaeelltch
 541 saeyvghlra tekmtrelh ressnfdsiy icpstfacaq latgaacrlv eavisgevin
 601 gaavvrppgh haeqdaacgf cfnsavava rhaqtisgha lrlividwv hngngtqhmf
 661 eddpsvlyvs lhrydhgtff pmgdegassq igrtaagtgtf vnvawngpm gdadylaawh
 721 rlvlpiayef npelv1vssag fdaargdplg gcqryspeya hlth1lmgl sgrillileg
 781 gynltsses maactrs1lg dpppl1lpr pplsgalassi tetiqvhrry wrs1rvtmkve
 841 dreppsskl vtkkapqapk prlaermtrr ekkvleamng kvtssasfgee stpgqtnset
 901 avvalcqdpq seaatggatl aqtiseaaig gamlgqtse eavggatpdq ttseetvrga
 961 ildqtseda vggatigqt seeavggatl aqtiseaame gatldqttse eapggtelq
 1021 tplasstdhq tpqispsstli gsr1rtlelgs esqgasesqa pgeenllgea
 1081 aggqdmadsm lmqgsrgltd qaiifyavtpl pwcpchlvac pipaagldvt qpcgdcgtiq
 1141 enwvc1scyq vycgryiingh mlqhnsgn plvlsyidls awcyyccqayv hqgalldvkn
 1201 iahqnkffged mphph (SEQ ID:11)

FIG. 6A

FIG. 6B-1

541	attgacctga	tggaaaacaa	ccagtaacat	aatgaggagg	aactccgtt	ccttagcagac
601	accacgact	cagtttatct	gcattccgaaac	tcataactcct	gtgcctcgct	ggcctcaggc
661	tctgtcctca	ggctgggtgga	tgcgggtctg	ggggctgaga	tccggAACGG	catggccatc
721	attaggccctc	ctggacatca	cggccagcac	agtcttatgg	atggctattg	catgttcaac
781	cacgtggctg	tggcagcccg	ctatgctcaa	cagaaacacc	ggacccggag	gttccttatac
841	gttagattggg	atgtgcacca	cggtaaggaa	acacagttca	ccttcgacca	ggaccccagt
901	gtcccttatt	tctccatca	ccgctacggag	cagggtaggt	tctggccca	cctgaaggcc
961	tctaactggg	ccaccacagg	tttcggccaa	ggccaaggat	ataccatca	tgtgcttgg
1021	aaccagggtgg	ggatggggaa	tggtaactac	attgtcttgtt	ttctggcacgt	cctgtgtggcc
1081	gtcgccctcg	aggccaggcc	tcagctggtc	ctggggccct	ctggatttga	tgcctgtggaa
1141	ggggaccccc	aggggagat	ggggcgccact	ccggcagggt	tgcccaggct	aacccacctg
1201	ctcatgggtc	tggcaggagg	caagctgatc	ctgttcttgt	agggtggcta	caacccctcgcc
1261	gccccggctg	aaggcgtcag	tgtttcgctc	cacacccctc	tggggacccc	ttggcccatg
1321	ccggaggcac	ctgggtcccc	ctggcggaggc	gcccaggctt	cgtttctgt	tgctcttgaaa
1381	gccccgtgg	ccttctggaa	ggtttcttgt	agatcaactg	agaccgttgg	gagggacaac
1441	atggggagg	acaatgtaga	ggagaggcggag	gaggaaggac	cctggggagcc	ccctgtgctc
1501	ccaatccctga	calggccagt	gctacagtct	cgcacaggcc	tggtctatga	ccaaaatatg
1561	atgaaatca	gcaacttgt	ggacagccac	cacccctgggg	tacccaggcg	catcttccatc
1621	atcatgtgcc	gtctggggaa	gctggggctt	gcccggggct	gttccaaactt	ttcccaatgg
1681	cctgcccacag	aggctggagct	gtctacctgt	cacagtgtctg	gttccaaactt	ttcccaatgg
1741	gcccacaaa	aaatggaaaac	ccggggaggctg	cacccgtggaga	gttccaaactt	ttcccaatgg
1801	tataatgtgc	ccagttacctt	cgccctgtcga	cagcttggca	ctggcgctgc	ctggccggctg
1861	gtggggggctg	tgcttctcagg	agagggtcctg	aatgggtctg	ctgtgtgtgg	tcccccaggaa
1921	caccacggag	aggaggatgg	agcttggggct	tttigtttt	tcaactctgt	ggctgtgggt
1981	gctcgccatg	cccagactat	cgtggggcat	gcccctacggaa	tctgtattgt	ggattttgggt
2041	gtccaccacg	gtatggaaac	tcaggcacatg	tttgaggatg	accccagggt	acctataatgt
2101	tccctggcac	gttatgtatca	tggcaccttc	ttccccatgg	gggatgggggg	tgccatggaa
2161	cagatcgccc	ggggccgggg	cacaggcttc	accgttccacg	tgcttcccat	ggggcccccggc
2221	atgggtgatg	ctgactacat	agctggctgg	cattggccatg	tgccctacgag	tgccctacgag
2281	tttaaccctag	aactgggtct	gggtctcaggt	gtgttttttgg	ggatccgggg	ggatccggctg

SUBSTITUTE SHEET (RULE 26)

2341 ggggctggc aggttccacc tgagggttat gccccacccccc cccacactca
 2401 gccagggtggc gcattttatcc tatccttagag gggtggctata acctgacatc
 2461 tccatggctg cctgcactcg ctccctcg gggagacccac caccctgtct
 2521 cggcccccac tatcaggggc cttggcctca atcactgaga ccatccaatg
 2581 tactggggca gcttacgggt catgaaggca gaaggacagg aaggacccctc
 2641 ttggtccacca agaaggcacc ccaaccaggc aaacccttagt tagctgagcg
 2701 cgagaaaaaa aggttctggg aggaggcatg gggaaagtca cctcgccatc
 2761 gagtcaccc caggccagac taactcagg acagctgtgg tggccctcac
 2821 ccctcagagg cagccacagg gggggccact ctggccaga ccattctga
 2881 ggggaggcca tgctggccca gaccacccca gaggaggctg tcggggggac
 2941 gagaccacct cagggggac tttttgggg gggccacgt gccattctgg accggacc
 2881 001 gctgttgggg gggccacgt gggccaggact acctcaggagg
 2881 061 ctggccaga ccatctggg gggggccatg gggaggccca
 2881 121 gagggggctc cagggggcac cgggtgtat caaactcc
 2881 181 gagcccccc caaccttacc tgttgaggaa actaccccc
 2881 241 attggggatc tcaaggactt ggggttaggc agcgaacctc
 2881 301 gccccaggag aggaaacctt accaggaggag gggggccctc
 2881 361 atgctgacgc agggatctag gggccctact gatcaggcca
 2881 421 ctggccctgt gtcccatcc gttggcagta tgccccatc
 2881 481 acccaaccc ttggggactg tggaaacaatc caagagaact
 2881 3541 cagggttacc ttgggttccatcaatggc cacatgttcc
 2881 3601 caccggctgg tcctcaggca catcgaccctg ttggccctat
 2881 3661 gtccaccacc aggttccct agatgtggaa aacatggcc
 2881 3721 gatatggccc acccaccta agccccaggaa tacggttcc
 2881 3781 gatagaccag ttccaggctg ttccaggctg tacctttggat
 2881 3841 atcccatctt gaatatctt tgcaactccc caagagggtct
 2881 3901 taagagaactt gggacgatata atttgtggatc tccccctggc
 2881 3961 ccactactcc agcccaggaa gaaagggggg cagctcagtg
 2881 4021 tcatgaggat aacattggcg ggaggggagt taactggcag
 2881 4081 taataaagta caagctgtt (SEQ ID NO: 12)

FIG. 6B-3

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1 mdlrvyqgrpp veppeptll alqrpqrllh hlflaglqqq rsvepmrlsm dtppmpe1qvg
61 pgeqe1rql1 hkdkskrssav assvvkqkla evilkkqqa lertvhpnsp gipyrtlepi
121 etegatrsml ssflppvpsi psdppehfp1 rktvsepnlk lrykpks1e rrknpl1lrke
181 sappslrrp aetlgdssps ssstpasgcs spndsehgpn pilgdsdr1t hpt1gprpi
241 lgsph1pt1f1 phglepeagg clpsrlqp1l 11dpsgshap lltvpg19pl pfhfaqsint
301 terlsgsglh wplsrtrsep lppsatapp pgpmqpr1eq lkthvqvikr sakpsekpr1
361 rqipsaede tdggggggvv ddglehrelg hgqpear1gpa plqghpqvii wedqrlagrl
421 prgstgdcv1 lplaqqghp lsraqsspa1 pasisapepa sqarvlssse tpart1pf1t
481 gliydsmlk hqcschgnsr hpehagriqs iwsrlqerg1 rsqceclrgr kasieelqsv
541 nserhv1lyg tnplsr1kld ngklagiaq rmfem1pcgg vgydtdtiwn elhssnaarw
60,1 aagsytdlaf kvasrelkng favrppghh adhsstagfc ffnsvaiacr q1qqqskask
661 askillivdwd vhngngtqqt fyqdpsvlyi slhrhddgnf fpqsgavdev gagsgegfnv
721 nvawaggldp pngdpeylaa frivmpiar efspd1lvls agffdaaeghp applgyhyvs
781 kcfgyntqql mnlaggav1 alleghdita icdaseacva allgnrvdpl seegwkqkpk
841 pqchp1sgr dpgaqq (SEQ ID NO:13)

FIG. 7A

FIG. 7B-1

1381	tggccatgg	gcagggccgag	cccaaggccc	ccgttcctct	ccaggcacac	cctcagggtgt
1441	tgctctggaa	acaggcaggca	ctggctggcc	ggctcccccg	ggggcaggacc	ggggacactg
1501	tgctgcttcc	tctggcccaag	ggggggcacc	ggcttcgttc	ccgggtctcg	tcttcccaag
1561	ccgaccctgc	ctcaactgtca	gcccaggaggc	ctggccaggca	ggcccccggatc	ctctccagct
1621	cagagacccc	tgccaggacc	ctgcccattca	ccacagggtt	gatctatgac	tgggtcatgc
1681	tgaaggacca	gtgctcctgc	ggtgacaaaca	gcaggcaccc	ggggcaggccc	ggccgcatcc
1741	aggcatctg	gtcccggtcg	caggagggg	ggcctcgagg	ccagtgttag	tgtctccgag
1801	gccggaaaggc	ctccctggaa	gagctgcgt	cggtccactc	tgaggcggcac	gtgctcctct
1861	acggcaccaa	cccgctcagg	cgcctc当地	tggacaaccc	tggactggca	ggggctccgg
1921	cacaggat	gtttgagatg	ctggccctgt	gtggggtttg	gttggacact	gacaccatct
1981	ggaatggat	tcatccllcc	aatgcagccc	gtggggccgg	tggcagtgtc	actgacactcg
2041	ccttcaaagt	ggcttctcg	gagtaaaa	atggtttccgc	tgtgggggg	ccccaggac
2101	accatgcaga	tcatcaaca	gccatgggt	tctgtttctt	caactcgtg	gccatcgcc
2161	gccggcagct	gcaacagcag	agcaaggcca	gcaaggccag	caagatcc	atgttagact
2221	ggggactggca	ccatggcaac	ggcacccagg	aaaccttcta	ccaaaggcccc	agtgtgtct
2281	acattcct	gcatggccat	gacggacggca	acttttccc	ggggagggtgg	gctgtggatg
2341	aggtagggcc	tggcaggcggt	gagggtctca	atgtcaatgt	ggcctggggt	ggagggtctgg
2401	accccccatt	gggggatctt	ggttacccct	ctgctttcag	gatagtcgtg	acgcccattcg
2461	cccggagggt	ctctccagac	ctagtcctgg	tgtctgccc	atttgtatgt	gctgggggtc
2521	acccggcccc	acgggtggc	taccatgttt	ctgccaatgt	ttttggatac	atgacggcagg
2581	aactgatgaa	cctggcaggaa	ggcgcaagtgg	tgctggccct	gggggggtggc	catgacactca
2641	cagccatctg	tgacggccct	ggggctgtg	tctgggttaac	agggtggatc	ctctctggag
2701	ccctttcaga	agaaggctgg	aaacagaaac	cccacaccta	atggccactcg	ggctggcatgc
2761	ggcggtatcc	gggtgcacag	taaatactgg	ggctggcatgc	aggccctggc	ctccctgtcca
2821	gactccctgg	tgcccttagatgt	ggccggggct	gacaaaaggaa	aaggggggc	agtggaggcc
2881	ctggcggtcc	tctctgtgg	catcctggct	gaaggataggc	cctcggagca	gctgggtggag
2941	gagggaaaggaa	ctatgaatct	ctaaaggctct	ggaaccatct	gccggccac	catggcccttg
3001	ggacccctggtt	ctcttctaacc	ccctggcaat	agccccat	cctgggtctt	tagagatct
3061	gtggggcaagt	agttggaaacc	agagaacaggc	ctgctgtgtt	tgacagttat	cccaggggaggc
3121	gtggggaaaat	c (SEQ ID NO:14)				FIG. 7B-2

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1 meepeepads gqslvpvyiy speyvsmcds lakipkrasm vhsliayal hkqmrvkpk
61 vasmeematf htdaylqh1q kvsqegdddh pdsieygl9y dcpategifd yaaaiaggati
121 taaqclidgm ckvainwsgg whakkeas gfcylndav1 gilr1rrkfe rilyvdld1h
181 hggvedafs ftskvmtvsl hkfsppffpg tgdvsdvglg kgryysvnvp iqdgigdeky
241 yqicesvlke vyqafnpkav vlqlgadtia gdpmcsfnmt pvgigkclky ilqwqlatli
301 1gggylnlan tarcwtyltg vilgktisse ipdbefftay gpdyvyleitp scrpdrneph
361 rigqilnyik gmlkhvv (SEQ ID NO: 15)

FIG. 8A

1 gaaattcggc acggactcg gccgaattcg gcacgagaac gttttaagg gaaaggatgg
 61 ggagccggag gaaccgggg acagtggca gtgcgttgtc cgggttata tctataggcc
 121 cgagtagtc agtatgttg actccctggc caagatccc aacgggcca gtatggtgc
 181 ttctttgatt gaaggcatatg cactgcataa gcaaatgggg atagtaagg ctaaagtggc
 241 ctccatggag gagatggcca cttccacac tgatgttat ctgcagcgc tcaggaaagg
 301 cagccaagg ggcatgtatg atcatccggc tcatacgaa tatggctat gttatgactg
 361 cccaggcact gaaggatat ttgactatgc agcagctata ggaggggcta cgatcacagg
 421 tgcccaatgc ctgattgacg gaatgtgcaa aatggatggcat aactggctg gagggggcca
 481 tcatgcaagg aaaggatggc catctggtt tcgttataatc aatgatgtcg tcctggaaat
 541 attacgatgg cgacggaaat ttgagggtat tccttacgtg gattcgatc tgccatgg
 601 agatgggtta gaagacggat tcagttcac tcggaaatgc atgaccgtgt ccctggcaca
 661 attctccca ggattttcc cggaaacgg tgacgtgtcc gacgtggcc tagggaaagg
 721 acggactac agtgttaatg tgcccatcca ggatggcata caagatggaaa aatattacca
 781 gatctggaa agtgtactaa aggaaatgtata ccaaggcctt aatccccaaag cagtggctt
 661 attctccca ggattttcc cggaaacgg tgacgtgtcc gacgtggcc tagggaaagg
 721 acggactac agtgttaatg tgcccatcca ggatggcata caagatggaaa aatattacca
 781 gatctggaa agtgtactaa aggaaatgtata ccaaggcctt aatccccaaag cagtggctt
 841 acagctggaa gcccacacaa tagctgggta tccatgtgc tcctttaaca tgactccagt
 901 gggaaattggc aagtgtctca agtacatccc tcaatggcag ttggcaacac tcatttcgg
 961 agggaggggc tataaccttgc caaacacggc tcgatgtctgg acataacttga cggggtcat
 1021 cctaggaaaa accatccatcc tggatgtcc ctttccatgg cttttcacaatgatggccat
 1081 tgattatgtg ctggaaatca cgcacaggctg ccggccagac tgggtctagt tgacagaaaag
 1141 ccaacaatc ctcaactaca tcaaaggaa tctgaagcat gatcatggatggccat
 1201 agatcagggtt tccagaggctt aggggtgg cctataatgg aagacaggctg ttatgcaag
 1261 cagtttgggg aatttgtgac tggaggaaa atttggaaa aattttcc tggggatggccat
 1321 caagggcat caagtggcag ctggcttcct ggggtggaaa ccacatggaaatgg
 1381 caactggacc tagggaaa agggatatac ccacatggaaatgg ccaggatcc
 1441 cacacacaca aatggaaatt ttaatctttg aaaattttt ttaaggcgaat tgggggggg
 1501 agtattttaa tcatcttgc tggaaacagat cagaaggcttgg atgaggaggcag tcaccatgg
 1561 tggggcagg aggcaactgtg caggcagggn tngggccctn ggaccancca ngtggggccc
 1621 tggggagagan ggtactgtatc ngcagactgg gagg (SEQ ID NO:16)

FIG. 8B

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lipo MM1 AS1 100nM

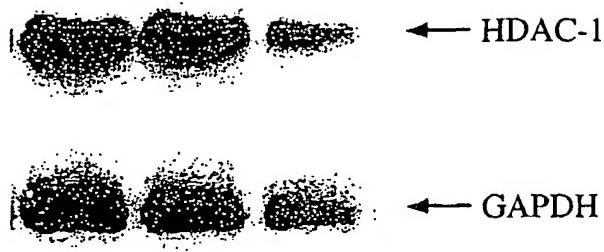


FIG. 9A

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lipo MM AS 100nM

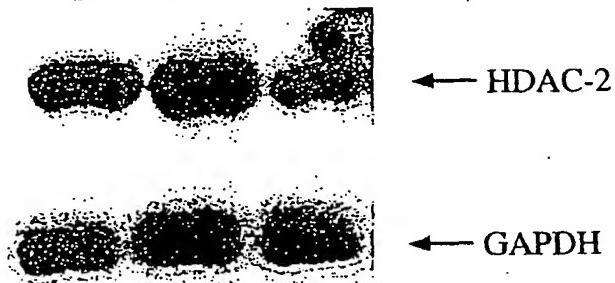


FIG. 9B

lipo AS MM 100nM

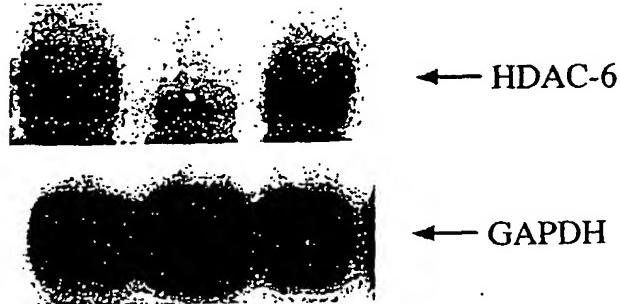


FIG. 9C

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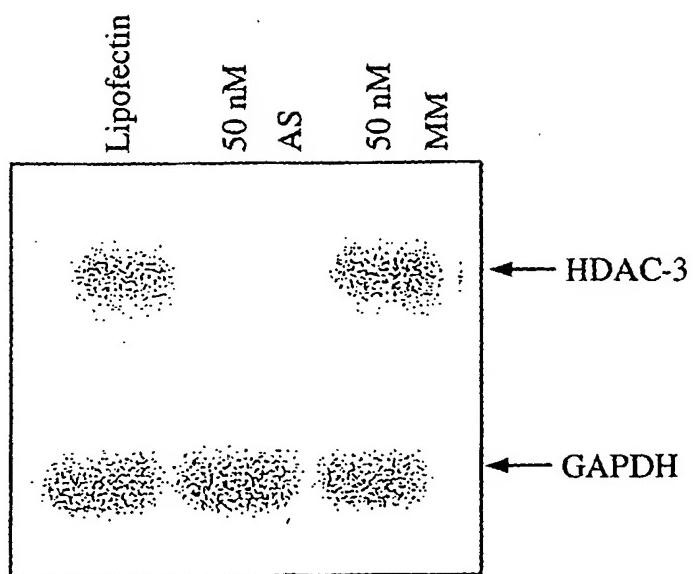


FIG. 9D

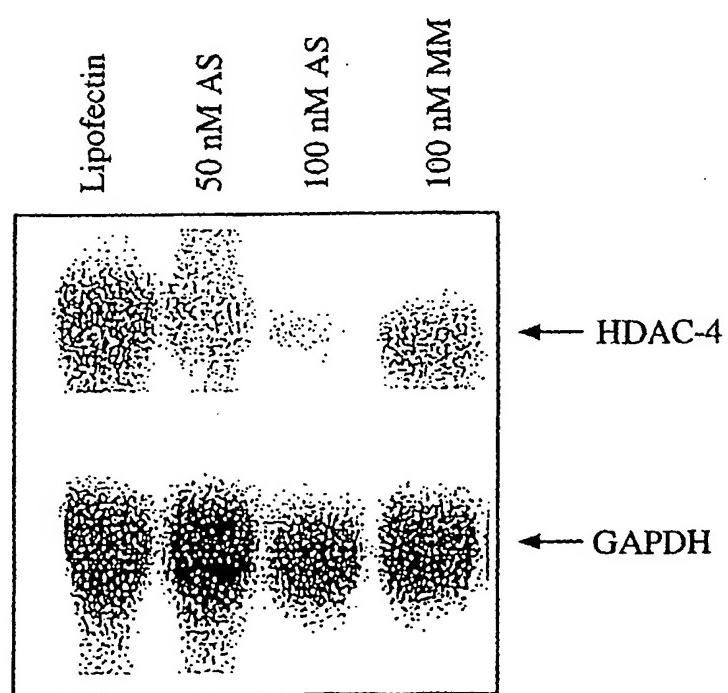


FIG. 9E

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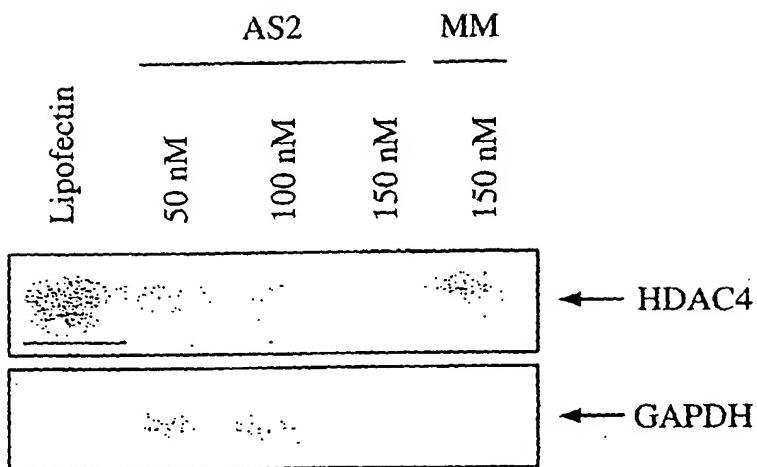


FIG. 9F

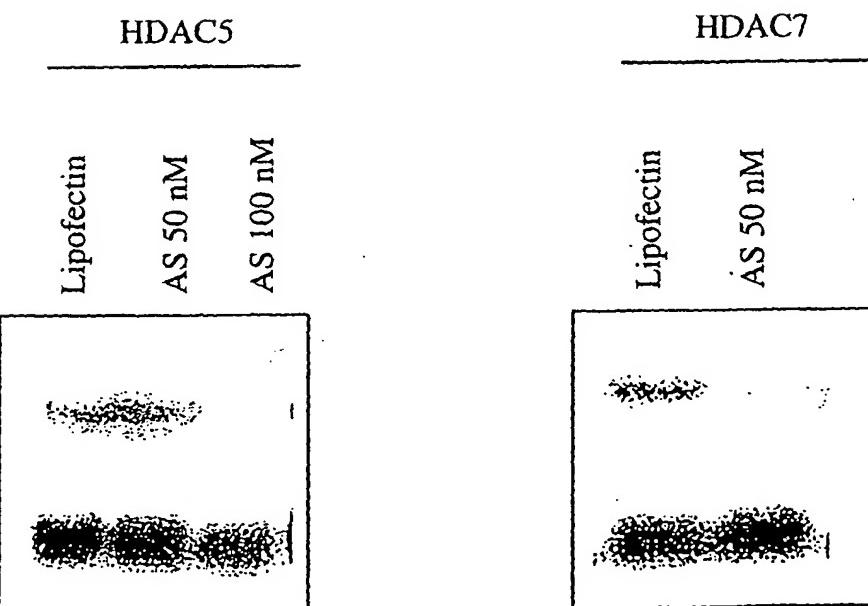


FIG. 9G

FIG. 9H

REF ID: A11 AB1 E 001

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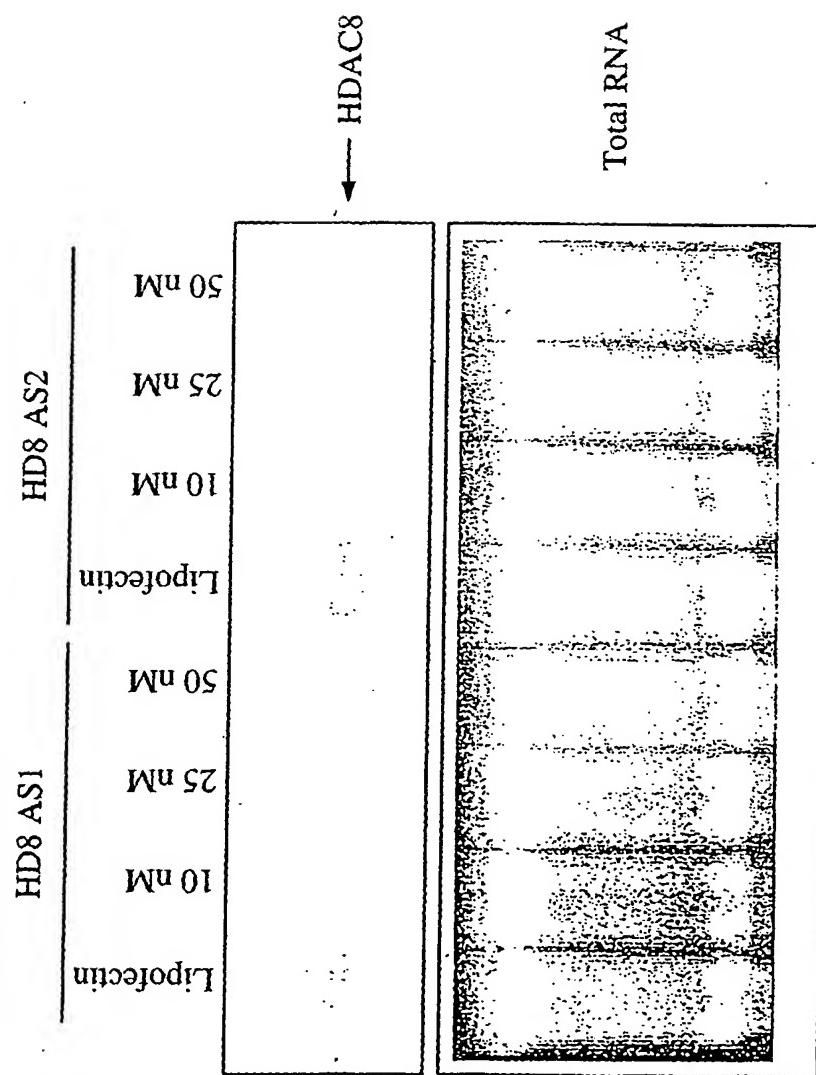
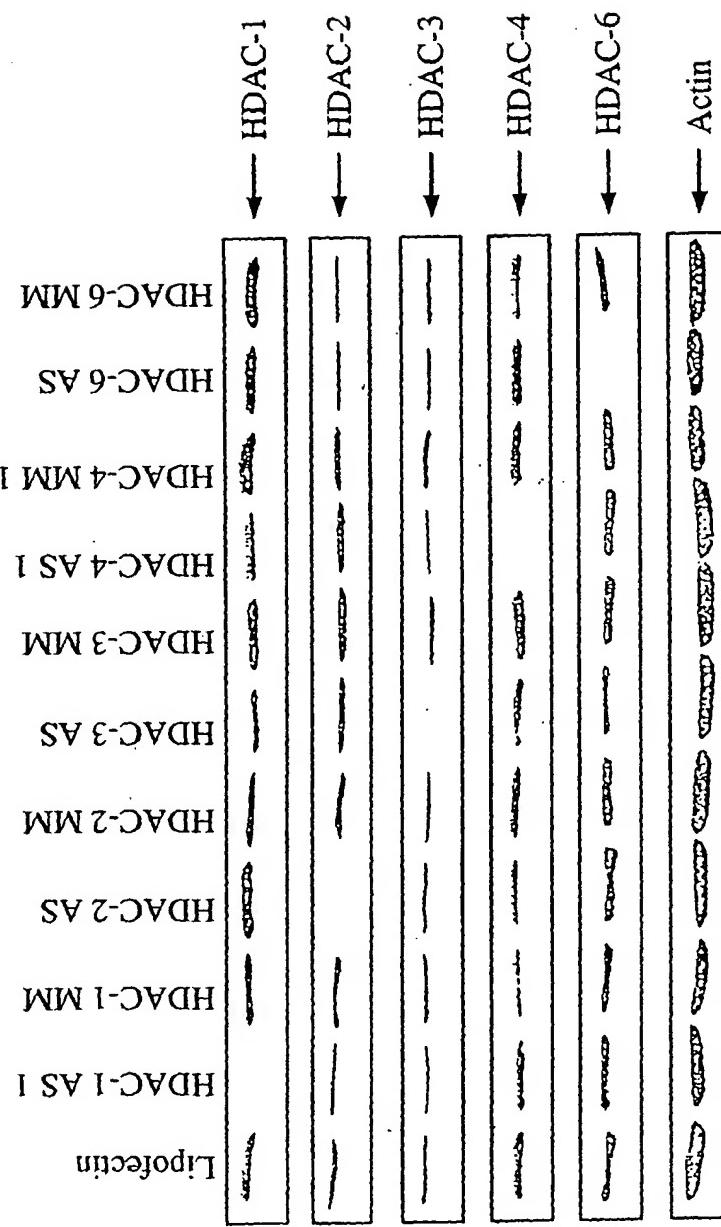


FIG. 9I

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AS = Antisense
 MM = Mismatch
 NS = Non-specific control

3 day treatment
 Oligonucleotide cone – 50nM

FIG. 10A

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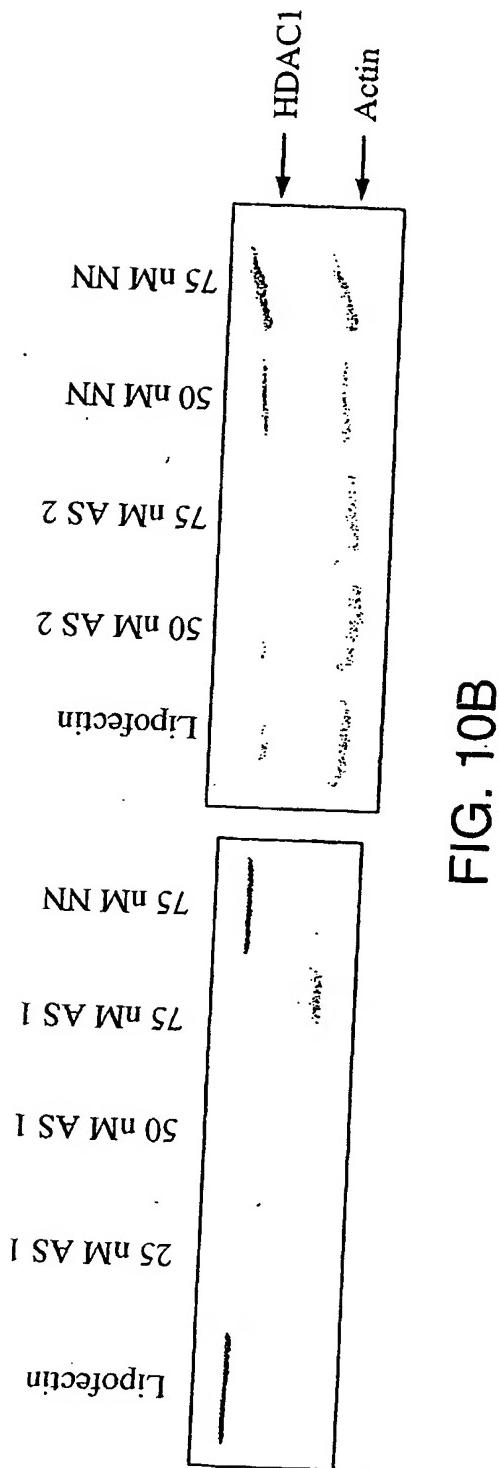


FIG. 10B

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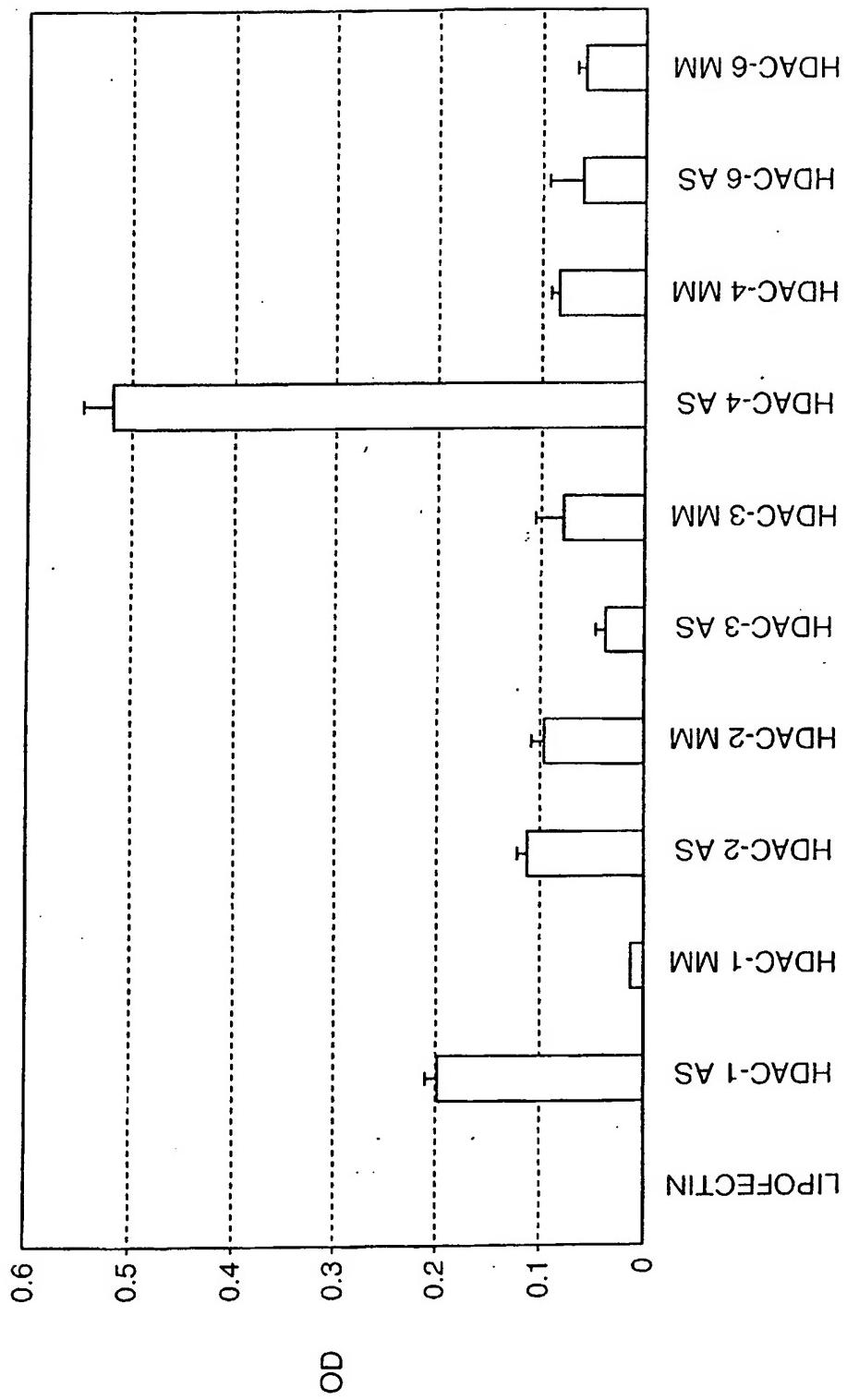


FIG. 11

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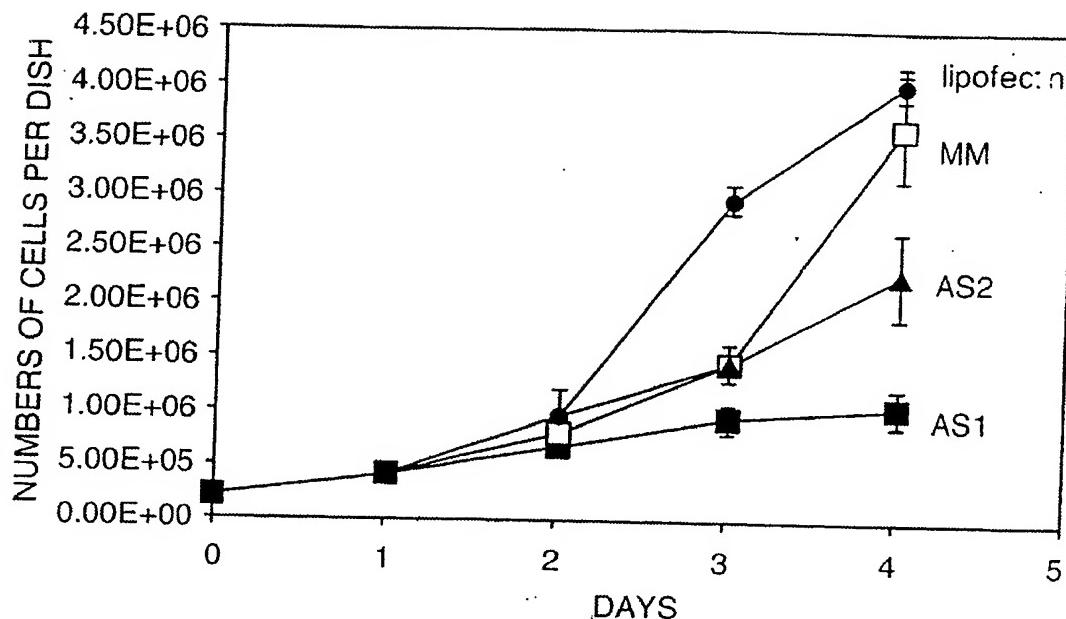


FIG. 12A

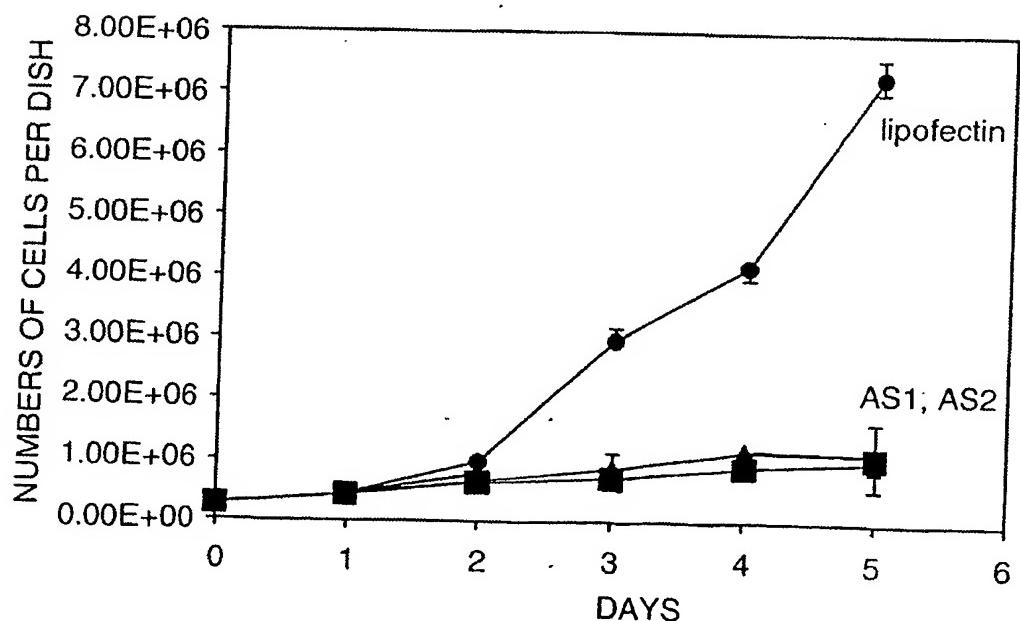


FIG. 12B

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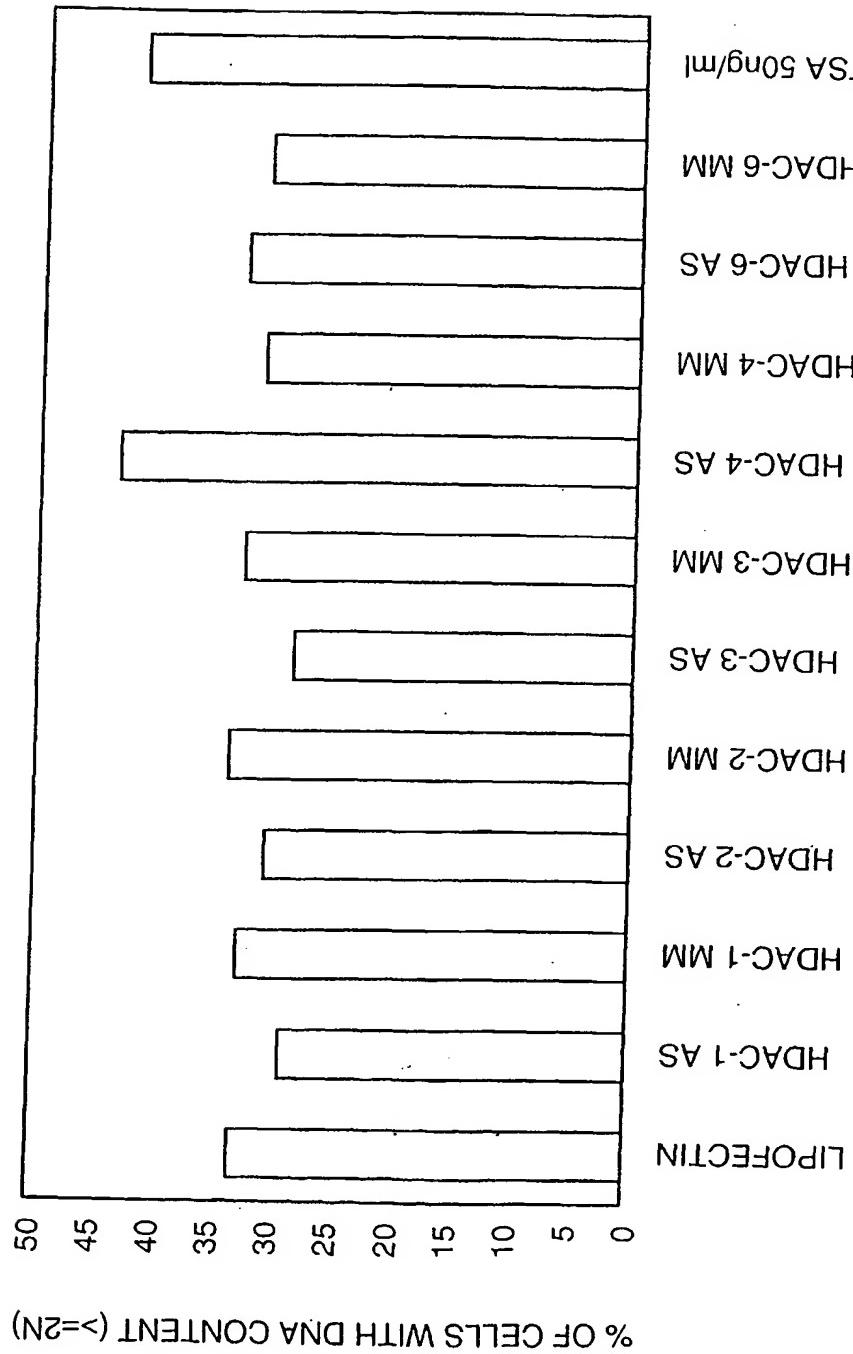


FIG. 13

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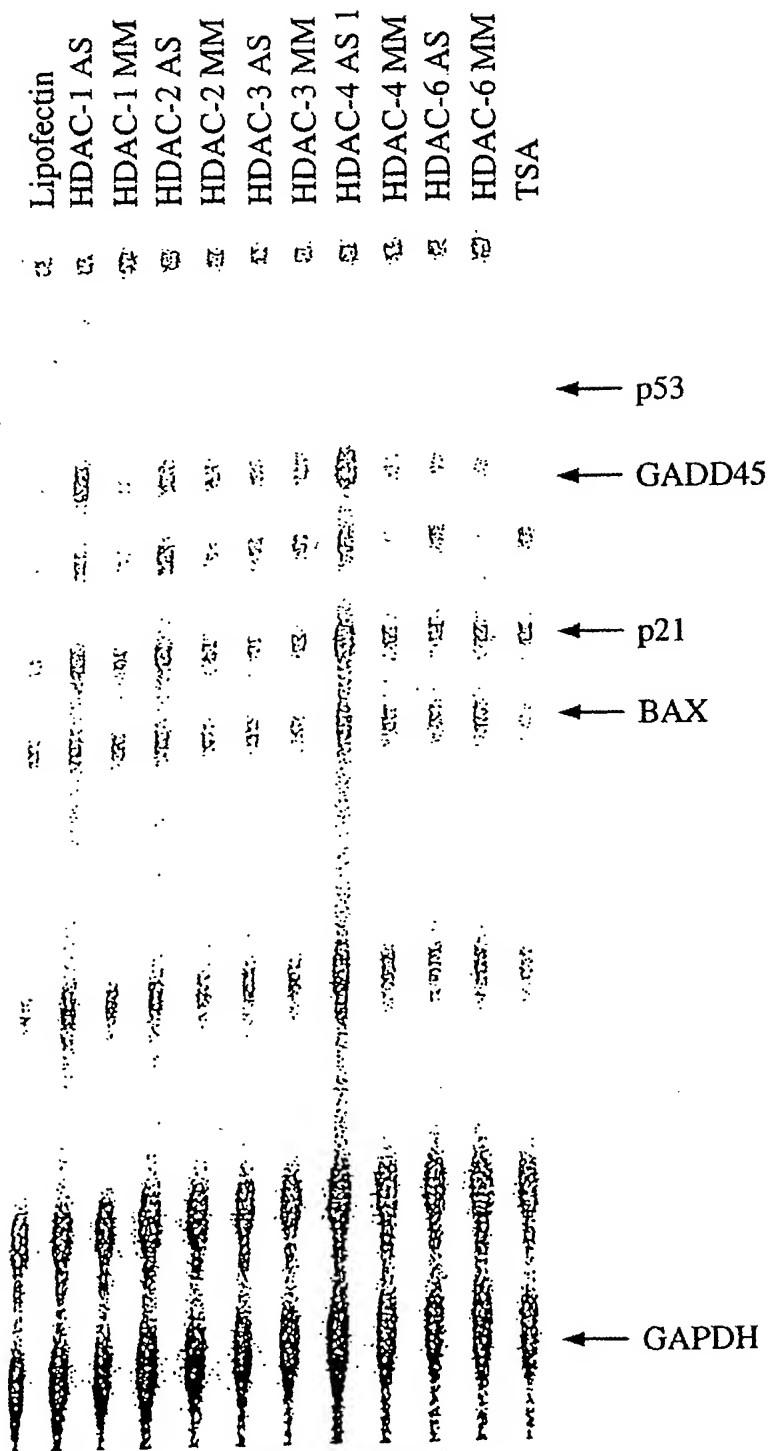


FIG. 14

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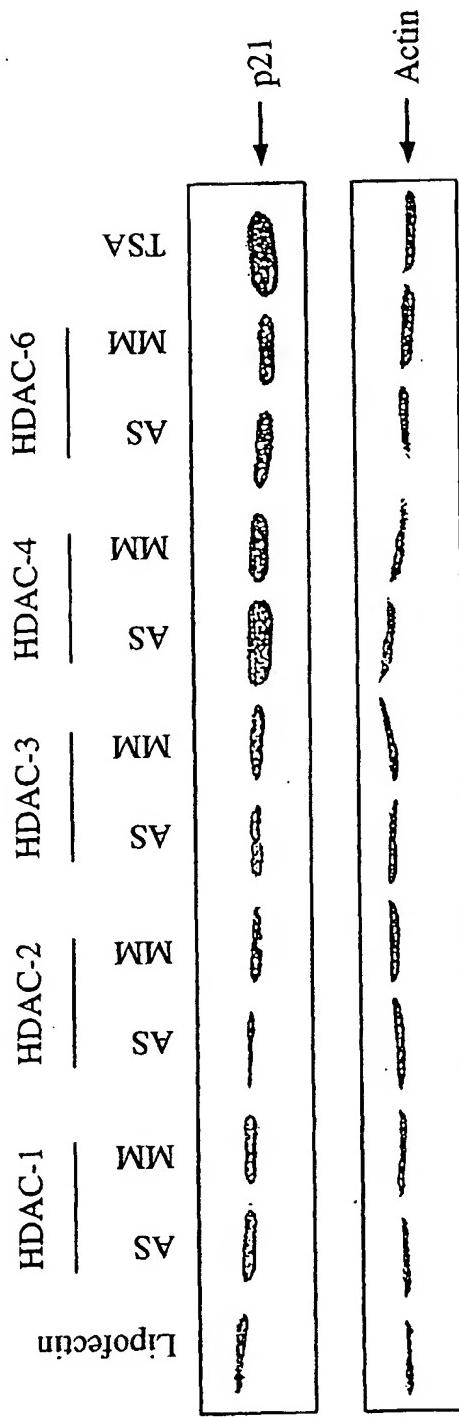


FIG. 15

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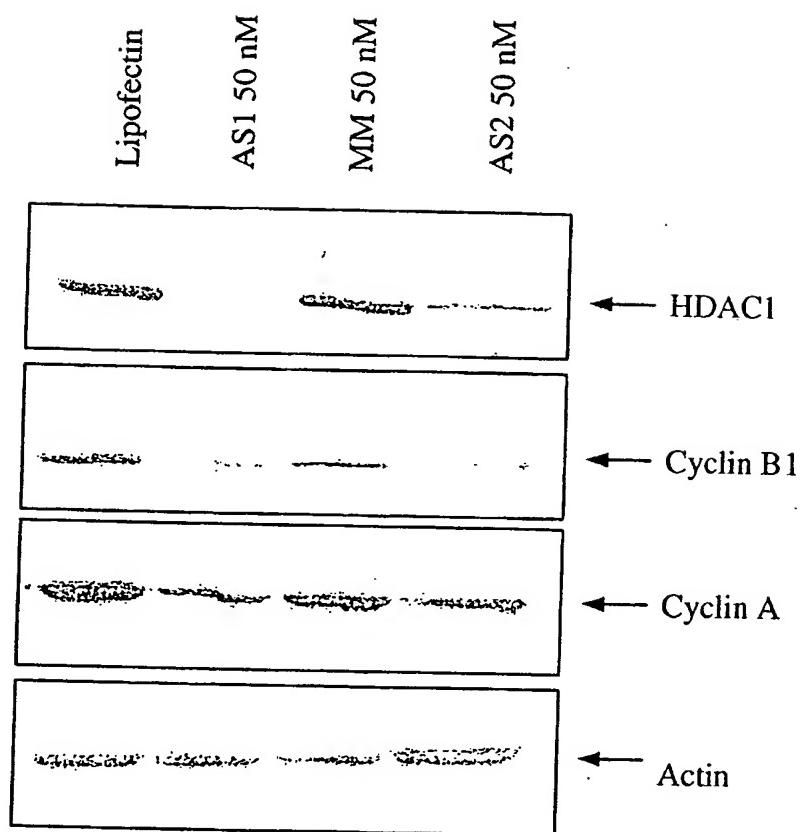
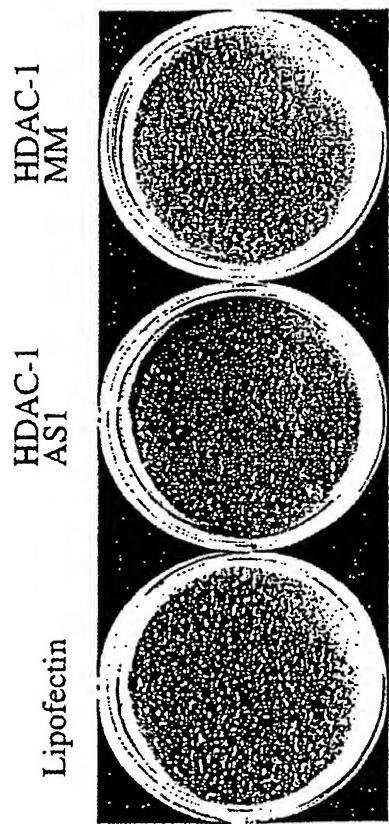


FIG. 16

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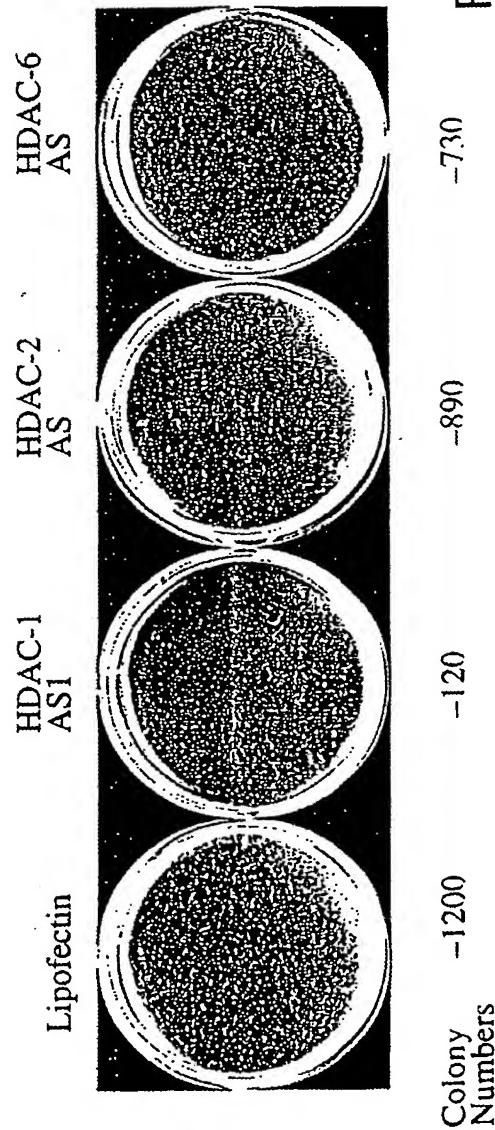
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FIG. 17A



Colony
Numbers

-1200 -1160 -120



Colony
Numbers

-890 -730 -120

FIG. 17B

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Organization
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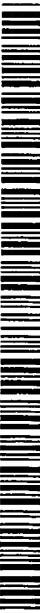
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 2003/006652 A3

(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

A. CLASSIFICATION OF SUBJECT MATTER	IPC 7 C12N15/11 A61K31/7125 C07H21/04	C12Q1/44 //A61P35/00
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12N A61K C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 97 35990 A (JAMISON TIMOTHY F ;HARVARD COLLEGE (US); TAUNTON JACK (US); HASSIG) 2 October 1997 (1997-10-02) page 5, line 8 -page 6, line 27 page 27, line 13 -page 29, line 2 page 48, line 15 -page 65 claims; examples</p> <p>---</p> <p>-/-</p>	1-3,6-8, 26-48

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

V document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2003	06/03/2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Andres, S

INTERNATIONAL SEARCH REPORT

PCT/IB 01/02907

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>YOSHIDA M ET AL: "POTENT AND SPECIFIC INHIBITION OF MAMMALIAN HISTONE DEACETYLASE BOTH IN VIVO AND IN VITRO BY TRICHOSTATIN A" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 28, 5 October 1990 (1990-10-05), pages 17174-17179, XP000616087 ISSN: 0021-9258 cited in the application the whole document</p> <p>---</p>	1,26,45
A	<p>ZHAO Q ET AL: "EFFECT OF DIFFERENT CHEMICALLY MODIFIED OLIGODEOXYNUCLEOTIDES ON IMMUNE STIMULATION" BIOCHEMICAL PHARMACOLOGY, vol. 51, no. 2, 26 January 1996 (1996-01-26), pages 173-182, XP000610208 ISSN: 0006-2952 the whole document</p> <p>---</p>	4,5,9
P,X	<p>WO 00 71703 A (METHYLGENE INC) 30 November 2000 (2000-11-30) the whole document</p> <p>---</p>	1-11, 26-48
P,X	<p>WO 00 23112 A (BESTERMAN JEFFREY M ;MACLEOD ALAN ROBERT (CA); METHYLGENE INC (CA)) 27 April 2000 (2000-04-27) examples 9,10 page 29; tables 2,3 claims 38-50</p> <p>---</p>	1-12, 26-37, 44-48
E	<p>WO 01 70675 A (METHYLGENE INC) 27 September 2001 (2001-09-27)</p> <p>page 46 -page 54; table 1 page 68; example 13 page 203 -page 223; examples 159-162 claims</p> <p>-----</p>	1-16, 24-37, 44-48

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 01/02907

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 26-33 (as far as in vivo methods are concerned) and claims 34-37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: 17-23
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-9,26-48 (all partially) and claims 10-11

An antisense oligonucleotide against HDAC1; modified forms thereof and its applications in therapy and diagnostic.

2. Claims: 1-9,26-47 (all partially) and claims 12-13

As for subject 1., but concerning HDAC2.

3. Claims: 1-9,26-47 (all partially) and claims 14-15

As for subject 1., but concerning HDAC3.

4. Claims: 1-9,26-48 (all partially) and claim 16

As for subject 1., but concerning HDAC4.

5. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC5.

6. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC6.

7. Claims: 1-9,26-47 (all partially)

As for subject 1., but concerning HDAC7.

8. Claims: 1-9,26-47 (all partially) and claims 24-25

As for subject 1., but concerning HDAC8.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17-23

The application as filed does not comprise claims 17 to 23. Consequently only claims 1-16 and 24-48 have been taken into account.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No
PCT/IB 01/02907

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9735990	A 02-10-1997	AU 2990597 A WO 9735990 A2		17-10-1997 02-10-1997
WO 0071703	A 30-11-2000	AU 6718200 A EP 1173562 A2 WO 0071703 A2 JP 2003500052 T		12-12-2000 23-01-2002 30-11-2000 07-01-2003
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